RETROVIRAL SUPERANTIGENS

By

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RETROVIRAL SUPERANTIGENS

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Superantigens (SAg) are potent inducers of T cell activation that cause proliferation and massive cytokine release. The receptors for superantigens on antigen-presenting cells (APC) are major histocompatibility (MHC) class II molecules. Both Nef protein of human immunodeficiency virus (HIV) and mouse mammary tumor virus (MMTV) SAgs are encoded in genes that overlap the terminal repeat at the 3' end. Using synthetic peptides, a region was identifed on the MMTV-1 SAg, corresponding to residues 76-119, that specifically binds to mouse MHC class II antigens. MMTVSAg(76-119) also bound to and competed with bacterial superantigens for binding to MHC class II peptides, suggesting similar binding regions on class II. These studies are the first demonstration that retroviral superantigens bind to MHC class II antigens.

A peptide corresponding to an internal region of HIV Nef protein was identified that specifically binds to to human class II antigens. Nef protein induced proliferation of human peripheral blood mononuclear cells (PBMC) in 85-90% of the HIV-negative donors tested. Nef stimulation required APC, and did not require processing. Interleukin 2 and gamma interferon were produced in Nef-stimulated cultures. These results strongly suggest that Nef acts like a retroviral SAg that stimulates $CD4^+$ T cells.

Activated T cells are required for HIV replication. The role of Nef in HIV pathogenesis was investigated by treating PBMC with Nef prior to *in vitro* infection with HIV. Significant levels of infection were found in these cultures, as compared to unstimulated controls. Nef-stimulated T cells were found to express T cell activation markers. Inactivated HIV-infected cells were capable of inducing proliferation in autologous fresh PBMC, and proliferation was significantly reduced by anti-Nef antibodies. These results indicate that Nef is expressed on the surface of infected T cells, possibly in the context of class II antigens, and as such, Nef can activate a cellular reservoir in an paracrine fashion for continual viral replication. Thus, Nef is an HIV-encoded SAg-like mitogen that promotes HIV replication in T cells. As such, Nef is probabaly an HIV virulence factor.

CHAPTER I
INTRODUCTION

Overview

A novel class of antigens called superantigens has recently been characterized. Unlike conventional antigens, which stimulate approximately 1 in 10,000 cells, superantigens can induce the proliferation and activation of as many as 1 in 5 T cells (Johnson et al., 1991). The staphylococcal enterotoxins, prototypic of this class of antigens, are among the most potent inducers of T cell proliferation, and are effective at concentrations as low as 10^{-16} M (Langford et al., 1978a).

Major differences exist between conventional antigens and superantigens. Superantigens exert their effects as whole molecules and do not require processing by antigen presenting cells for T cell recognition. Although superantigens require MHC class II molecules, they bind at site(s) outside the peptide antigen binding groove (Russell et al., 1990; Dellabonna et al., 1990). Superantigens presented in the context of MHC class II molecules are recognized by the variable region of the β chain (Vβ) of the T cell receptor. Only T cells that express T cell receptors with specific Vβ regions will be activated by a given superantigen. For example, toxic shock syndrome toxin-1 activates only Vβ2-bearing human T cells (Abe et al., 1992) and staphylococcal enterotoxin A activates several Vβs on human T cells, including Vβ5 and Vβ12, (Kappler et al., 1989). Thus, T cell responses to superantigens are said to be Vβ-specific.

T cell activation by superantigens results in massive proliferation of Vβ-specific T cells. Concomitant with proliferation is prodigious production of cytokines, in particular interleukin 2 and gamma interferon (IFNγ). Such responses can be detrimental to the host, as is the case with food poisoning which occurs upon ingestion of staphylococcal enterotoxin-laden food (Bergdoll, 1985). As an eventual consequence of superantigen activation, T cells can become unresponsive to further stimulation (anergy) or undergo programmed cell death (apoptosis) (Kawabe and Ochi, 1990, 1991; Rellahan et al., 1990). Thus, the fate of T cells activated by superantigen differs from that of cells activated by conventional antigens, with the possible consequence of loss of immune function.

Superantigens are produced by a number of microorganisms, ranging from bacteria to viruses. The prototypic bacterial superantigens are the enterotoxins produced by *Staphylococcus aureus* (Table I). Staphylococcal enterotoxin A (SEA) is the most common cause of food poisoning, and its superantigen effects on immunocytes have been implicated in the syndrome of short-term nausea, fever, and diarrhea that are symptomatic of food poisoning (Bergdoll, 1985; Johnson and Magazine, 1988). Another staphylococcal enterotoxin, toxic shock syndrome toxin-1 (TSST-1), is produced during infection and its superantigenic effects result in toxic shock syndrome (Bergdoll, 1985). Other bacterial sources of superantigens are the group A streptococci, which produce pyrogenic exotoxins that have been implicated in psoriasis (Kotzin et al., 1993) and rheumatic heart disease (Lewis et al., 1993). Superantigens may also be involved in tuberculosis (Ohmen et al., 1994) and Reiter's syndrome (Uchiyama et al., 1993). One species of mycoplasma, *M. arthiditis*, has been shown to produce a superantigen that may be involved in arthritis (Cole and Griffiths, 1993).

Table I.
List of bacterial superantigens implicated in disease.

Organism	Superantigenic Protein	Disease/Effect[a]
Prototype:	**Staphylococcal enterotoxins**	
Staphylococcus aureus	Enterotoxins	Food poisoning
		Toxic shock
		Kawasaki's disease (?)[b]
		Multiple sclerosis
(?)		
Group A streptococci	Pyrogenic exotoxins	Psoriasis (?)
		Rheumatic heart disease (?)
Mycoplasma arthitidis	T-cell mitogen	Arthritis (?)
Mycobacterium tuberculosis	Not identified	Tuberculosis (?)
Yersinia pestis	Not identified	Reiter's syndrome
		Reactive arthritis
Clostridium perfringens	Exotoxin	Sudden infant death syndrome (?)

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[a] Johnson et al., 1995.

[b] Denotes diseases in which superantigens have been implicated, but no direct evidence is available.

Like their bacterial counterparts, viruses produce superantigens that have been implicated in pathogenic processes (Table II). The prototypic viral superantigen is that produced by the oncornavirus, mouse mammary tumor virus (MMTV) (Marrack et al., 1991; Frankel et al., 1991; Woodland et al., 1991; Dyson et al., 1991). The Gag protein of mouse leukemia virus, the causative agent of mouse acquired immunodeficiency syndrome (MAIDS), has been shown to possess superantigenic properties (Hugin et al., 1991). A superantigen has been implicated in the pathogenesis of rabies virus (Lafon et al., 1992) and recently the superantigenic viral component has been identified as the nucleocapsid protein (Lafon et al., 1994). It has been suggested that human immunodeficiency virus (HIV) encodes for a superantigen that may, at least in part, be responsible for the immunopathogenesis associated with infection (Laurence et al., 1992). Recent evidence points to a superantigen encoded by human cytomegalovirus (CMV) that expands specific Vβ-bearing T cells, thereby enhancing HIV replication in CMV/HIV-infected individuals (Dobrescu et al., 1995). Although several reports suggest that other viruses, such as Epstein-Barr virus and human foamy virus, encode for superantigens, to date no hard evidence has been obtained.

The possible harmful consequences of activation by superantigens make this class of molecules relevant to human disease. The prodigious expansion of T cells having diverse specificities may be important in the induction and establishment of autoimmunity. Concomitant cytokine production may also have deleterious effects. The potential for loss of immune function via mechansisms such as anergy or apoptosis can also result in immunodeficiency diseases. Superantigens have been shown to be encoded by retroviruses, suggesting that they may play a role in the syndrome associated with human

Table II.
List of viral superantigens implicated in disease.

Virus (Family or Subfamily)	Superantigenic Protein	Disease/Effect[a]
Prototype: Superantigen from Mouse Mammary Tumor Virus		
Mouse mammary tumor virus (oncornavirus)	*vsag* gene product	Mammary tumors
Mouse leukemia virus (oncornavirus)	Gag protein	Murine AIDS
Human immunodeficiency virus (lentivirus)	Not identified	AIDS
Human foamy virus (?)[b] (spumavirus)	*bel3* gene product	Grave's disease
Rabies (rhabdovirus)	Nucleocapsid	Rabies
Epstein-Barr virus (?) (herpesvirus)	Not identified	B cell lymphoma Chronic fatigue syndrome (?)
Cytomegalovirus (herpesvirus)	Not identified	Enhanced HIV replication

[a] Johnson et al. 1995.

[b] Denotes diseases in which superantigens have been implicated, but no direct evidence is available.

immunodeficiency virus. Thus, superantigens may be involved in disease processes and, as such, can be classified as virulence factors.

Bacterial Superantigens

In the 1970s, staphylococcal enterotoxins were recognized as inducers of lymphocyte proliferation (Peavy et al., 1970). Responses to staphylococcal enterotoxin A (SEA) stimulation were shown to be T cell specific (Johnson and Bukovic, 1975), with high levels of gamma interferon (IFNγ) produced by this lymphocyte fraction (Langford et al., 1978a). SEA was shown to have mitogenic effects on human lymphocytes at extremely low concentrations, making it one of the most potent T cell activators known (Langford et al., 1978a). The ability to produce large quantities of IFNγ using SEA paved the way for the characterization of the key immunomodulatory properties of this cytokine (Johnson, 1985). Interestingly, intravenous administration of SEA to mice resulted in the induction of suppressor cell activity that was present in spleen as early as 24 hours after injection (Johnson, 1981; Torres et al., 1982). SEA-induced suppressor cells were capable of blocking naive spleen cells from responding to SEA stimulation. Thus, SEA was shown to stimulate T cells to proliferate and produce cytokines, and to induce suppressor cell activity.

Further investigations on the interaction of bacterial enterotoxins with human lymphocytes revealed that antigen-presenting cells are required for proliferative effects and that binding to major histocompatibility (MHC) class II antigens occurred (Carlsson et al., 1988; Fleischer and Schrezenmeier, 1988; Mollick et al., 1989). Unlike most antigens which interact with MHC class II molecules as peptides, the staphylococal enterotoxins were shown to bind in an unprocessed form (Fleischer and Schrezenmeier, 1988). The site of interaction of enterotoxins on the MHC class II molecule was shown to be distinct from conventional antigens in that binding occurs distal to the peptide antigen binding groove (Russell et al., 1990; Dellabonna et al., 1990). Thus, this class of antigens differs significantly from classic antigens and were given the designation of superantigens (Marrack and Kappler, 1990).

The study of staphylococcal enterotoxins has led to a wealth of information on the interaction of superantigens with MHC class II molecules. Through the use of synthetic peptides, regions of interaction of SEA and MHC class II antigens have been identified. Regions on the SEA molecule that are involved in binding to MHC class II molecules on antigen presenting cells include the N-terminus and three internal sequences (Griggs et al., 1992). A peptide corresponding to the N-terminus of SEA, SEA(1-45), blocked proliferation of cells in response to whole native SEA (Pontzer et al., 1990). Interestingly, a peptide corresponding to another of these MHC class II-binding regions, SEA(121-149), has agonist properties in that it induces cytokine production (Pontzer et al., 1993) and proliferation (A.C. Hobeika, personal communication). Conversely, sites on MHC class II molecules which bind superantigens have been identified. Regions on mouse MHC class II molecules that interact with SEA have been identified and include the α-helical region of the β chain associated with the peptide binding groove encompassed by amino acids 65-85 (Russell et al., 1991). A similar site has been found on human MHC class II antigens (Herman et al., 1991). A superantigen-binding region on the α helical region of the α chain associated with the peptide binding groove of MHC class II has also been defined (Russell et al., 1991). Both of these binding sites reside outside of the peptide binding groove and are required for SEA-induced mitogenesis. Thus the two outside faces of the α helices of the peptide binding groove are involved in binding of SEA.

Presentation of superantigen in the context of MHC class II molecules is required for interaction with T cell receptor (TCR). For this reason superantigen sites that interact with TCR have been more difficult to elucidate. However, through the use of synthetic peptides, a TCR site that interacts with SEA has been identified. A peptide corresponding to residues 57-77 of mouse Vβ3 blocked SEA-induced proliferation and IFNγ production (Pontzer et al., 1992). Vβ3-bearing mouse T cells are known to be stimulated by SEA. Future studies may help determine the sequences involved in the formation of the ternary complex of superantigen:MHC:TCR. Such studies are needed to determine how superantigens activate T cells bearing specific Vβs and induce anergy and deletion of these Vβ subsets.

Retroviruses

Retroviruses belong to a family of viruses that are characterized by the use of a unique RNA-dependent DNA polymerase, reverse transcriptase. Reverse transcriptase, in conjunction with another virally-encoded enzyme, RNAse H, transcribes the single-stranded RNA genome into a double-stranded linear DNA provirus. This DNA intermediate is then capable of integrating into the host genome. Retroviruses were first described in studies in which cell-free filtered extracts were shown to transmit sarcoma to chickens (Rous 1910, 1911). Since that time, retroviruses have been found in several vertebrates including mice, cats, and primates. The first human retrovirus to be discovered was human foamy virus, which has been speculated to cause disease (Achong et al., 1971), although definitive proof of pathogenicity is lacking. In 1980, the causative agent of adult T-cell leukemia was discovered, human T-cell leukemia virus (Poiesz et al., 1980; Yoshida et al., 1982), and established this family of viruses as human pathogens.

The retrovirus family consists of three subgroups based on similar pathogenicities: oncornaviruses, lentiviruses, and spumaviruses. Spumaviruses or foamy viruses are the least characterized of the three subfamilies of retroviruses, and have been grouped according to their ability to cause vacuolation of infected cells *in vitro*, which gives the cells a "foamy" appearance. Spumaviruses have been found in primates and humans, and are generally considered benign, although data suggest these viruses may play a role in human disease. Oncornaviruses are tumor-causing retroviruses, and members of this subfamily include the avian leukosis-sarcoma viruses, mouse mammary tumor virus, feline leukemia virus, and human T-cell leukemia virus. As a result of proviral integration into the host genome, tumors arise by upregulation of host genes that encode for growth factors or by retroviral-encoded oncogenes.

Viruses belonging to the lentiviral subfamily include the "slow" viruses maedi/visna and equine infectious anemia virus. More recently, the viruses that cause human and feline acquired immundeofiency syndromes have been classified as lentiviruses, based on several parameters including genomic complexity and virion morphology. Unlike oncornaviruses, lentiviruses have not been implicated directly in causing neoplastic disease. Members of the lentiviral subfamily cause long-term disease characterized by autoimmunity, encephalopathy, immunodeficiency, or a combination thereof. Lentiviruses are considerably more complex than some of the other retroviral subfamilies, in that the level of gene regulation is much greater.

Mouse Mammary Tumor Virus Superantigen

The first viral superantigens to be described are the proteins encoded by the open reading frame that overlaps the 3' long terminal repeat of the oncornavirus, mouse mammary tumor virus (MMTV) (Marrack et al., 1991; Frankel et al., 1991; Woodland et al., 1991; Dyson et al., 1991). Although MMTV superantigens were recognized in the 1990s, they were originally described by Festenstein in the early 1970s as minor lymphocyte stimulating (mls) antigens (Festenstein, 1973). The ability of T cells from certain mouse strains to be stimulated by lymphocytes from MHC-identical strains was ascribed by Festenstein to the presence of mls antigens. More recently, mls antigens were found to be endogenous superantigens from germline-encoded MMTV provirus (Choi et al., 1991; Acha-Orbea et al., 1991). Like their bacterial counterparts, MMTV superantigens are thought to be presented in the context of MHC class II antigens. MMTV superantigens are known to stimulate T cells in a Vβ-specific fashion (Acha-Orbea and Palmer, 1991). MMTV superantigens show MHC preference, with more efficient presentation occurring in the context of I-E as opposed to I-A, although presentation by I-A does occur (MacDonald et al., 1989). Interestingly, this MHC preference corresponds with the greater infectivity of B cells bearing I-E (Held et al., 1994b).

MMTV is a type B retrovirus that was determined to be the causative agent for the induction and transmission of mammary carcinomas in mice (Heston et al., 1945). It has been known for several years that infectivity by MMTV requires an intact immune system (Tsubura et al., 1988), and only recently has this paradox been explained (Held et al., 1993, 1994a). Transmission of MMTV to offspring can occur via infectious virions in mother's milk or can occur vertically as endogenous provirus. Most mouse strains have been shown to contain one or more copies of endogenous MMTV, and many contain distinct MMTV strains (Salmons and Gunzburg, 1987). Upon passage into the gut of the host, virus enters the gut-associated immune tissue and infects B cells. B cells then express the MMTV-encoded superantigen, presumably in the context of class II antigens, causing Vβ-specific T cell stimulation. Although investigators have been able to show that actual infection of T cells occurs, it is thought that T cell stimulation and subsequent cytokine production by these cells indirectly enhances the further infection of B cells (Held et al., 1994a). Infected B cells migrate to the main site of viral infection, the mammary gland. Epithelial cells then become infected and are the source of infectious virions that are transmitted in milk (Ringold, 1983). Proviral integration can occur during infection of mammary epithelial cells, resulting in tumors.

Germline-encoded MMTV serves an important protective mechanism for the host. It is known that bacterial superantigens can cause anergy and/or deletion of Vβ-specific T cells (Johnson et al., 1992). Similarly, expression of MMTV superantigen early in the ontogeny of the immune system induces the eventual deletion of T cells bearing Vβs specific for that particular strain of MMTV superantigen. In this manner, T cells that would otherwise be stimulated are lost and the host is protected against subsequent infection by MMTV strains that stimulate those Vβ-specific T cell populations. This has been shown in mice transgenic for a MMTV superantigen that stimulates $V\beta14^{+}$ T cells (Golovkina et al., 1992; Acha-Orbea, 1991a). Transgenic mice showed partial to complete deletion of $V\beta14^{+}$ T cells, depending on the level of superantigen expression. Those mice in which $V\beta14^{+}$ T cells were deleted were subsequently protected from infection upon challenge with the same MMTV strain.

Evidence for a Superantigen Associated with Human Immundeficiency Virus

In the early 1980s, unusual incidences of young homosexual males stricken with *Pneumocystis carinii* pneumonia and Kaposi's sarcoma were reported to the Centers for Disease Control. These reports led to the first description of acquired immunodeficiency syndrome (AIDS). Subsequently, similar immunodeficiency-associated illnesses were reported among hemophiliacs, recipients of blood and blood components, and intravenous drug users and their heterosexual partners. The incidence of AIDS in these distinct populations was suggestive of an infectious agent, and this was confirmed when, in 1984, human immunodeficiency virus (HIV) was isolated from the blood of an AIDS patient (Klatzmann et al., 1984; Gallo et al., 1984). With the advent of diagnostic assays, the number of infected individuals was found to exceed the number of patients afflicted with AIDS, indicative of a serious epidemic. The number of deaths is expected to increase, as asymptomatic HIV-infected individuals progress to AIDS.

HIV is a member of the lentivirus subfamily of retroviruses. Infection with HIV is associated with a debilitating and eventually fatal immunodeficiency. In addition to immunologic impairment, neurological dysfunctions can occur, including encephalopathies, sleep disorders, and dementia (the latter is also referred to as HIV-associated cognitive/motor complex).

The size of the HIV genome is approximately 9.8 kbases. The genome encodes for a total of 16 proteins, some of which are post-translationally cleaved by either cellular or viral proteases. The primary HIV transcript is gag-pol mRNA, which is translated to yield Gag (group antigen) and Pol (polymerase). Synthesis of Gag-Pol occurs at a ratio of 20:1 (Oroszlan and Luftig, 1990). Gag is proteolytically cleaved by a viral-encoded protease, yielding 5 distinct proteins. Cleavage of Pol results in 2 proteins, one of which is reverse transcriptase. Two envelope (Env) proteins are the cleavage products from an initial Env precursor. In this case, the precursor is cleaved by a cellular protease. Two regulatory proteins, Tat (transactivator) and Rev (regulator for viral expression) are translated from multiply-spliced mRNA transcripts.

One putative regulatory protein, Nef (negative factor) is encoded by a gene that overlaps the terminal repeat at the 3' end of the HIV genome. It was initially named based on its apparent negative effects on viral transcription and replication *in vitro* (Terwillegar et al, 1986). However, subsequent reports have been conflicting, and have suggested that Nef has no effect (Hammes et al., 1989) or a positive effect (DeRonde et al., 1992) on HIV transcription. Such widely disparate Nef effects may, in part, be explained by the fact that several immortalized human T cell lines and primary human lymphocytes were used in these studies. These data may reflect inherent differences in primary cells and cell lines, such as activation signals. Data from studies on Nef-transfected T cells suggest that Nef may play a role in down-regulation of the CD4 molecule (Garcia and Miller, 1991; Mariani and Skowronski, 1993).

HIV-1 Nef is a 25-27K protein and is myristylated at the N-terminus. Myristylation is thought to be a mechanism by which Nef associates with the cytoplasmic membrane (Franchini et al., 1986; Guy et al., 1987). Nef primarily localizes to the cytoplasm (Franchini et al., 1986) but has been shown to be present on the surface of infected cells (Fujii et al., 1993). Antibodies to Nef appear early in HIV-infected individuals (Allan et al., 1985) and cytotoxic T lymphocyte activity has been detected against cells presenting Nef peptides (Bahraoui et al., 1990). These data confirm that Nef is expressed during active HIV infection.

A hallmark of infection with HIV is the alteration of CD4/CD8 T cell ratios due to loss of CD4$^+$ T helper cells. Such losses ultimately result in the inability of an infected individual to mount effective immune responses against opportunistic infections, thus resulting in death. Several possible mechanisms by which CD4 loss occurs have been postulated, including direct cytolysis by HIV (Lemaitre et al., 1990), HIV-induced syncitia formation (Lifson et al., 1986; Sodroski et al., 1986) and/or cytolytic T cell activity against infected CD4 cells (Zinkernagel and Hentgartner, 1994). In addition to skewing of the CD4/CD8 ratios, other immunologic alterations seen in HIV-infected individuals include polyclonal activation of B cells with increased immunoglobulin production and reduced antigen and mitogen responses (Edelman and Zolla-Pazner, 1989). Initial increased natural killer cell activity is observed in asymptomatic HIV-infected individuals, but these activities decrease during disease progression (Edelman and Zolla-Pazner, 1989).

Because of the known induction of T cell anergy and/or deletion by superantigens (Johnson et al., 1992), it has been speculated that the HIV genome encodes for a superantigen that may cause some of the immunopathologies observed with AIDS. Upon interaction with MHC class II antigens and TCR, Vβ-specific T cell populations would be activated and expand, eventually leading to functional (anergy) or actual (deletion) loss of T cells. Several points of evidence suggest a role for an HIV-derived superantigen. Although initial studies suggested that AIDS patients had deletions in Vβ T cell populations (Imberti et al., 1991), such deletions could not be found by others (Laurence et al., 1992). Vβ12+ T cells were shown to support enhanced replication of HIV compared to other Vβ subsets and proliferated in response to cells from HIV+ patients, suggesting the presence of a superantigen (Laurence et al., 1992). Asymptomatic patients exhibit altered

Vβ profiles (Dalgleish et al., 1992) as well as skewed T cell Vβ usage upon stimulation with staphylococcal enterotoxins *in vitro* (Bisset et al., 1993). Interestingly, one study found skewed Vβ T cell profiles in monozygotic twins that were discordant for HIV infection, with perturbations in several Vβs (Rebai et al., 1994). This last study is of particular importance in that the altered Vβ profiles in infected individuals cannot be ascribed to differences in MHC, since only identical twins were used. Thus, these studies hint at the presence of a superantigen encoded by HIV that may play a role in HIV pathogenesis by augmenting a population of T cells, resulting in a reservoir for virus replication, with eventual deletion of those T cells.

CHAPTER II
MMTV SUPERANTIGEN BINDING TO MHC CLASS II ANTIGENS

Introduction

SEA is one of the most potent T-cell mitogens known, and has been classified as a bacterial superantigen based on its ability to stimulate Vβ-specific T-cell subsets (Johnson et al., 1991). Concurrent with studies on SEA, minor lymphocyte stimulating (mls) antigens have recently been shown to be products of MMTV (Choi et al., 1991; Acha-Orbea et al., 1991). Two exogenous strains of MMTV encode for retroviral superantigens in genes that overlap the terminal repeat at the 3' end of the viral genome (Pullen et al., 1992; Choi et al., 1992). These genes have been designated *vsag*, denoting that they encode for viral superantigens (Marrack and Kappler, 1990). No direct binding of the putative MMTV superantigen to either MHC antigens or TCR has been shown. To determine sites that interact with class II MHC antigens, overlapping synthetic peptides were synthesized that encompass the putative extracellular domain of MMTV-1 superantigen. The data presented here indicate that a site that is encompassed by amino acid residues 76-119 of MMTV-1 superantigen competes with SEA for binding to class II MHC antigens. Further, direct binding studies show that this region of the MMTV superantigen binds directly to class II MHC antigens. These data indicate that SEA and MMTV superantigen share at least one common binding region on class II MHC molecules.

Materials and Methods

Synthetic Peptides

Overlapping peptides corresponding to the putative extracellular domain of the MMTV-1 vSAg protein (Pullen et al., 1992) were synthesized with a Biosearch 9500AT automated peptide synthesizer using N-(9-flurenyl)methoxycarbonyl chemistry (Griggs et al., 1992). Peptides were synthesized based on a surface profile which uses a composite of three parameters: 1) HPLC hydophilicity; 2) accessibility; and 3) segmental mobility (B value) (Parker et al., 1986). The sequences of the peptides are listed in Table III. Peptides were cleaved from the resins using trifluoroacetic acid/ ethanedithiol/ thioanisole/ anisole at a ratio of 90:3:5:2. The cleaved peptides were then extracted in ether and ethyl acetate and subsequently dissolved in water and lyophilized. Peptides were extensively dialyzed against water to remove the remaining cleavage products. Amino acid analysis of the peptides showed that the amino acid contents and molecular weights corresponded closely to theoretical values. Peptides were not purified further since reverse phase HPLC analysis of crude peptides indicated one major peak in each profile.

Cell Lines and Reagents

The A20 cell line (ATCC, Gaithersburg, MD) was used for MMTV superantigen binding studies. A20 cells are a BALB/c B lymphoma line that expresses Ia. Highly purified SEA and other staphylococcal enterotoxins were obtained from Toxin Technology (Sarasota, FL). Several monoclonal antibodies (mAb) were used in this study. MAbs were purchased from Accurate Chemicals, Westbury, NY. MAb K^d (34-1-2S) is specific for m31 determinant of K^d (Ozato et al., 1982). MAb IA^d (MK-D6) is specific for the $\beta 1$

Table III.
Amino acid sequences of vSAg peptides

vSAg Peptide	Sequence
vSAg(76-119)	DSFNNSSVQDYNLNDSENSTFLLGQGPQPTSSYKPHRLCPSEIE
vSAg(117-147)	EIEIRMLAKNYIFTNETNPIGRLLIMMLRNE
vSAg(142-172)	MMLRNESLSFSTIFTQIQRLEMGIENRKRRS
vSAg(166-195)	ENRKRRSTSVEEQVQGLRASGLEVKRGKRS
vSAg(190-222)	KRGKRSALVKIGDRWWQPGTYRGPYIYRPTDAP
vSAg(220-250)	DAPLPYTGRYDLNFDRWVTVNGYKVLYRSLP
vSAg(245-276)	LYRSLPFRERLARARPPWCVLSQEEKDDMKQQ
vSAg(274-313)	KQQVHDYIYLGTGMIHWKVFYNSREEAKRHIIEHIKALP
vSAg(76-119) (scrambled)	PNSNEGLSQQSTDPSPHNFILSNENSYPCYSLLGDVQREDSTKF

The vSAg(76-119) scrambled peptide sequence was generated using the sequence edit program (Devereax et al., 1984).

helix of I-A^d (Lee and Watts, 1990). All mAb were used at a final concentration of 10 μg/ml. MAbs used in this study had similar potencies as determined by the Hybridoma Core Facility, Interdisciplinary Center for Biotechnology Research, University of Florida. Polyclonal antibodies were used at a final dilution of 1:100. Poly IA^d (Cat.Y1-9-04-26-01) is specific for the m11 determinant of I-A^d and poly Ia.7 (Cat.Y1-7-02-02-01) is specific for the m7 determinant of I-E^d. Both antibodies were obtained from NIAID, Rockville, MD. Poly I-A^k was obtained from Accurate Chemicals, Westbury, NY.

Radioiodinations

SEA (2.5 μg) and vSAg peptide (10 μg) were radiolabeled using chloramine T as described (Griggs et al., 1992). Briefly, ligands were labeled with 500 μCi of Na^{125}I (15 mCi/μg, Amersham Corp., Arlington Heights, IL) in 25 μl of 0.5 M potassium phosphate buffer, pH 7.4, and 10 μl of chloramine T (5 mg/ml) for 2 min. After neutralization of the reaction with 10 μl each of sodium bisulfite (10 mg/ml), potassium iodide (70 mg/ml), bovine serum albumin (BSA; 20 mg/ml), and 15 μl of NaCl (4 M), the preparation was sieved on a 5 ml Sepharose G-10 column. The two fractions with the highest radioactivity in the first eluted peak were pooled and used in the radiolabeled binding assays. The specific activities of the SEs and vSAg peptide ranged from 130-150 μCi/μg and 30-60 μCi/μg.

Binding Studies

In binding studies using A20 cells, unlabeled competitors (SEs and vSAg peptides) were added in 50 μl volumes (in PBS with 1% BSA) at indicated final concentrations to 50 μl of cells in "Eppendorf" tubes. Competitors were incubated with cells at room temperature for 45 minutes, followed by the addition of radiolabeled SEs or vSAg peptide. After 45 minutes, the cells were washed three times and the radioactivity associated with the cell pellets was quantified using a gamma counter. L cells were used as an MHC class II-negative control cell line.

L cells were grown to confluence in the wells of microtiter plates and were subjected to the same incubation times and volumes of competitors and labeled ligands as used for A20 cells. After three washes, the cells were solubilized in 1% SDS, the liquid was absorbed by cotton-tipped applicators, and radioactivity was quantified using a gamma counter.

Radioimmunoassay

Two MHC peptides I-Aβb(30-60) and I-Aβb(60-90) were used to for binding of SEA and ORF(76-119) to class II antigens. The I-Aβb(60-90) site was previously shown to be involved in SEA binding to MHC class II antigens (Russell et al., 1990). MHC peptides were dissolved in PBS at a concentration of 25 μg/ml. Peptide solutions were pipetted into polystyrene plastic tubes and tubes were placed at 10°C for 4 hours to allow adherence of the peptides. The tubes were washed three times with PBS. Nonspecific sites on the plastic were blocked using 2 ml of PBS containing 1% BSA at 10°C overnight. After the tubes were washed three times of PBS containing 1% BSA, unlabeled competitors (SEA and vSAg peptide) were added in 0.1 ml and allowed to bind at room temperature for 3 hours. The tubes were washed three times with 2 ml of PBS/1% BSA prior to addition of 0.1 ml of either radiolabeled SEA or radiolabeled vSAg(76-119)peptide, at final concentrations of 2 nm and 5 nm, respectively. Radiolabeled ligands were allowed to bind to MHC peptides at room temperature for 4 hours. After washing three times with 2 ml of PBS containing 1% BSA, the tubes were placed in a gamma counter and bound radioactivity was quantified. Experiments were performed at least four times each using replicates of three.

Results

Competition of vSAg Peptides with Radiolabeled SEA for MHC Binding

Overlapping peptides (referred to as vSAg peptides) corresponding to the predicted extracellular domain of the MMTV-1 superantigen were synthesized and their sequences are listed in Table III. The vSAg peptides were initially tested at a concentration of 200 μM for their relative abilities to compete with ^{125}I-SEA for binding to A20 cells, which express I-A^d and I-E^d (which are MHC class II antigens of the *d* haplotype, A isotype and E isotype, respectively). The vSAg(76-119) peptide reduced ^{125}I-SEA binding to A20 cells by 63%, while the other peptides had no effect (Figure 1). Thus, only one of the overlapping vSAg peptides, vSAg(76-119), significantly blocked the binding of ^{125}I-SEA binding to A20 cells.

vSAg(76-119) Peptide Competition Is Dose-Dependent

Dose response studies were performed on A20 cells with several vSAg peptides, including vSAg(76-119). The vSAg(76-119) peptide reduced ^{125}I-SEA binding by 50% at a concentration of 20 μM (Figure 2). Further, vSAg(76-119) peptide consistently competed with ^{125}I-SEA in a manner similar to unlabeled SEA, although SEA was 20 times more effective. Unlabeled SEA at a concentration of 1 μM reduced ^{125}I-SEA binding to A20 cells by 50%. These data are consistent with the reported K_d for SEA binding to I-E^d of approximately 10^{-6} M (Lee and Watts, 1990). The peptide corresponding to a

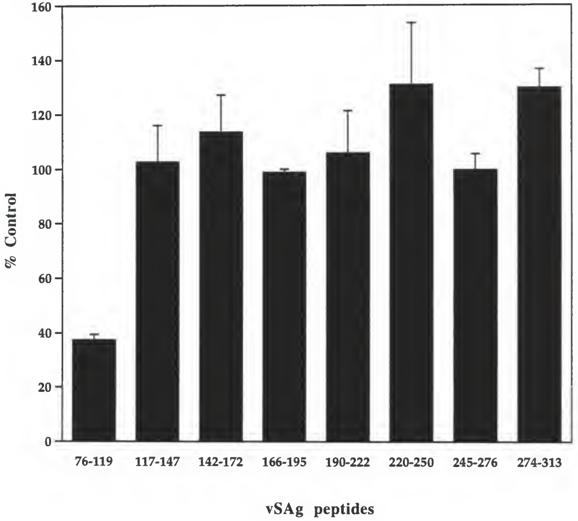

Figure 1. Ability of vSAg peptides to compete with ^{125}I-SEA for binding to A20 cells. Binding of ^{125}I-SEA in the absence of competitors was 6384±400 CPM. The data presented represent the mean of three individual experiments each performed in duplicate. Each bar represents the mean percent of SEA control binding in the presence of vSAg peptides ± SD.

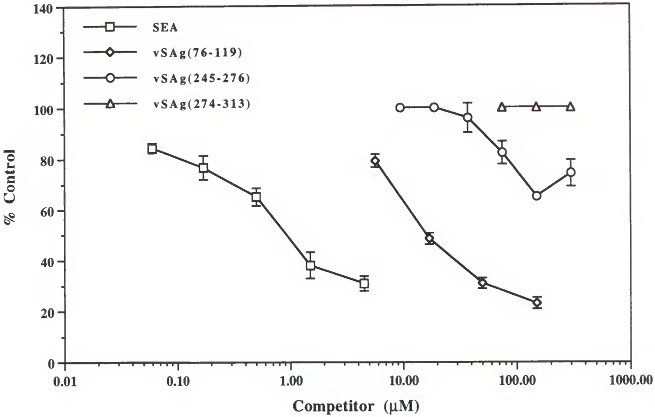

Figure 2. Ability of vSAg(76-119) to compete with ^{125}I-SEA for binding to A20 cells in dose response studies. ^{125}I-SEA was used at 2.5 nM. Binding of ^{125}I-SEA to A20 cells in the absence of competitors was 5184±380 CPM. The data presented represent the mean of three individual experiments, each performed in duplicate. Each point represents the mean percent of SEA control binding in the presence of competitors ± SD.

C-terminal region of the MMTV superantigen, vSAg(245-276), was not a consistent competitor for SEA binding. The peptide corresponding to the C-terminal tail, vSAg(274-313), did not compete. A peptide having the same amino acid content as vSAg(76-119) but in a scrambled sequence (see Table III for sequence) did not compete, thus indicating that vSAg(76-119) peptide competition was sequence specific. No direct binding of ^{125}I-vSAg(76-119) to SEA or to related superantigens was detected (data not shown). Thus, the vSAg(76-119) peptide binds specifically to A20 cells, and competes for SEA binding in a dose-dependent manner.

Direct Binding of vSAg(76-119) Peptide to A20 Cells

In direct binding studies, ^{125}I-vSAg(76-119) peptide bound to A20 cells and was effectively inhibited by both unlabeled SEA and unlabeled vSAg(76-119) peptide (Figure 3). Unlabeled SEA and unlabeled vSAg(76-119) peptide competed with ^{125}I-vSAg(76-119) in a similar manner, although SEA was a more potent competitor. SEA reduced ^{125}I-vSAg(76-119) binding by 50% at a concentration of 1.8 μM as compared to 25 μM for unlabeled vSAg(76-119) peptide. Neither vSAg(76-119) scrambled peptide nor the C-terminal vSAg peptide competed, thereby showing that vSAg(76-119) binding was sequence and region specific. TSST-1 did not compete with ^{125}I-vSAg(76-119) peptide for binding to A20 cells, while SEB competed less effectively than SEA, which is consistent with their relative abilities to compete with SEA for MHC class II binding (Fraser, 1989; Pontzer et al., 1991). Thus these results indicate that binding of vSAg(76-119) peptide occurs to murine MHC class II antigens. Binding of vSAg(76-119) may occur at a region(s) to which SEA also binds or to a neighboring site which interferes with the binding of SEA.

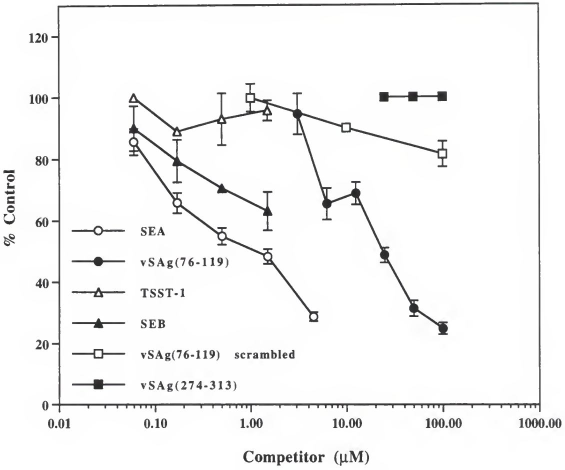

Figure 3. Ability of SEA, SEB, and TSST-1 to compete with ^{125}I-vSAg(76-119) for binding to A20 cells. Experimental conditions were the same as those described in Figure 1. ^{125}I-vSAg(76-119) was used at a final concentration of 2.3 nM. Binding of ^{125}I-vSAg(76-119) in the absence of competitors was 2278±224 CPM. The data presented represent the mean of three individual experiments, each performed in duplicate. Each point represents the mean percent of vSAg control binding in the presence of competitors ± SD.

Evidence that vSAg(76-119) Binds Specifically to MHC Class II Antigens

Two further pieces of evidence indicate that vSAg(76-119) binds to class II MHC antigens. Binding of vSAg(76-119) to class II negative mouse L cells was insignificant as compared to mouse A20 cells even at concentrations as high as 5 nM (Figure 4A). Monoclonal antibodies to class I MHC antigens had no effect on ^{125}I-vSAg(76-119) binding to A20 cells (Figure 4B). Polyclonal anti-I-A^{d} and anti-I-E^{d} significantly blocked binding and, in combination, these antibodies reduced binding to A20 cells by 73%. These data suggest that vSAg(76-119), like SEA (Lee and Watts, 1990), binds to both I-A and I-E. An I-A^{d} β1 helix-specific monoclonal antibody reduced binding by approximately 30%, suggesting that this is a region on I-A to which vSAg(76-119) binds. Polyclonal antibodies to I-A^{k} had minor effects on vSAg(76-119) binding. Thus, these data indicate that vSAg(76-119) binds to class II MHC antigens.

In order to directly determine if vSAg(76-119) binds to the β1 helix of I-A, a competitive radioimmunoassay was performed in which ^{125}I-SEA and ^{125}I-vSAg(76-119) were tested for their relative abilities to bind to I-Aβ^{b}(60-90) peptide. SEA and vSAg(76-119), but not scrambled VSAG(76-119), competed with both ^{125}I-SEA and ^{125}I-vSAg(76-119) for binding to I-Aβ^{b}(60-90) in a manner similar to the competition seen on whole cells (Figure 5A). As previously shown for SEA (Russell et al., 1990, 1991), vSAg(76-119) did not directly bind to I-Aβ^{b}(30-60) (Figure 5B). Thus, SEA and vSAg(76-119) bind to a similar region on the β chain of the class II MHC molecule.

Discussion

To date, no information has been available on the ability of MMTV vSAg protein to bind to MHC antigens and, in fact, this ability has been questioned (Marrack and Kappler, 1990; Acha-Orbea and Palmer, 1991; Acha-Orbea et al.,

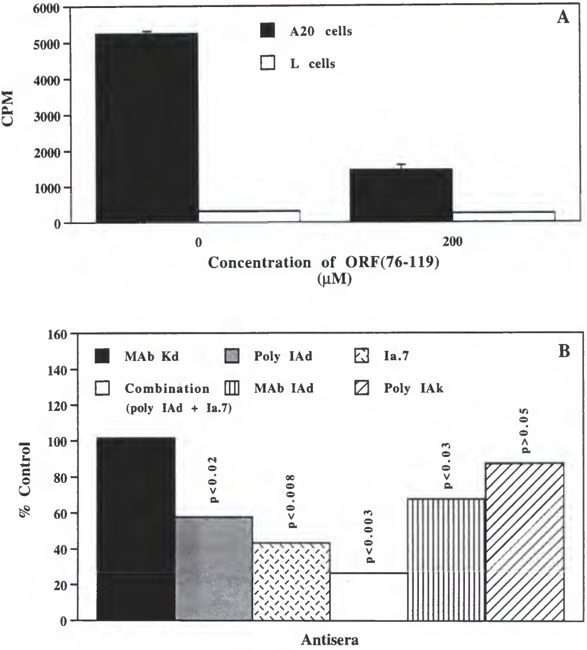

Figure 4. Evidence that vSAg(76-119) peptide binds to MHC class II antigens, and does not bind to class I antigens.

Panel A: Binding of ^{125}I-vSAg(76-119) to class II-positive A20 cells and class II-negative L cells. ^{125}I-vSAg(76-119) was used at a final concentration of 5 nM.

Panel B: Blockage of ^{125}I-vSAg(76-119) binding to A20 cells by antibodies to class I and class II MHC antigens. ^{125}I-vSAg(76-119) was used at a final concentration of 5 nM. MAbs were used at a final dilution of 1:30.

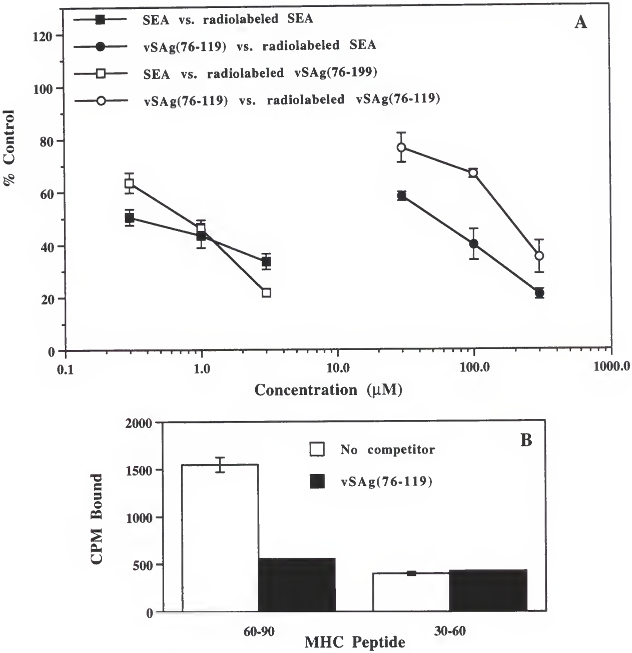

Figure 5. Binding of ^{125}I-vSAg(76-119) to MHC class II peptides.
Panel A: Binding of ^{125}I-SEA and ^{125}I-vSAg(76-119) to I-Aβ^b(60-90) and inhibition by vSAg(76-119). Each point represents the mean percent reduction of control binding in the presence of competitors ± SD. Binding of ^{125}I-SEA and ^{125}I-vSAg(76-119) in the absence of competitor was 2656±92 CPM and 1547±33 CPM, respectively.
Panel B: Relative ability of vSAg(76-119) to bind I-A^b(60-90) and I-Aβ^b(60-90) in the presence and absence of vSAg(76-119). Unlabeled and ^{125}I-vSAg(76-119) were used at final concentrations of 5 nM and 300 μM, respectively.

1991). Such binding studies are hampered by the difficulties inherent in expressing the MMTV superantigen. A model has recently been proposed that suggests that MMTV superantigens may act in the same or similar fashion to bacterial superantigens by bridging MHC antigens and TCR on the appropriate cell types (Acha-Orbea and Palmer, 1991). MMTV superantigens, presented in the context of MHC class II antigens, may act by stimulating and expanding Vβ-specific T cell subsets, thereby indirectly enhancing the further infection of B cells (Held et al., 1994a). The results presented in this chapter suggest that MMTV vSAg protein binds to MHC antigens, thereby strengthening the argument that bacterial and retroviral superantigens act in a similar manner.

It has been reported that MMTV superantigen is a 45 kd Type II integral membrane protein with an intracellular N-terminus, an essential hydrophobic transmembrane region near the N-terminus (residues 45-64), and a glycosylated extracellular C-terminus (Choi et al., 1992). Although no direct evidence exists, the variability of the C-terminal residues of vSAg proteins of various MMTV strains seems to correlate with their differences in Vβ specificity, lending support to the concept that this region binds TCR (Pullen et al., 1992). Truncated versions of the *vsag* gene were transfected into MHC class II-bearing cells and tested for superantigen activity. Complete loss of superantigen activity occurred when the MMTV *vsag* gene was N-terminally truncated to the third methionine (residue 122) and beyond (Choi et al., 1992). The authors concluded that a hydrophobic region, which was missing in this N-terminally truncated version, acts as a transmembrane region and is essential for superantigen activity. However, our binding data suggest that loss of superantigen activity may have been due, at least in part, to the deletion of the MHC-binding domain which is encompassed by residues 76-119. In fact, the superantigen activity of MMTV vSAg protein was lost in truncations that did not contain the region encompassed by residues 76-119.

Subsequent studies by the same group of investigators suggest that MMTV-7 superantigen is synthesized as a precursor molecule, is proteolytically cleaved at an internal site (~ residue 164) and is expressed as an 18.5 kd surface protein consisting of the C-terminal residues (Winslow et al., 1992). Membrane association of this truncated form may involve "tethering" to MHC class II antigens or to the N-terminal portion of the precursor molecule. As a cautionary note, the transfected cell line used to characterize this truncated form of the MMTV-7 superantigen was shown to have high expression of the protein but low activity. Conversely, a transfected cell line with moderate superantigen expression and high activity was not used for characterization.

Recently, studies were reported on the binding of truncated forms of MMTV-7 superantigen to human MHC class II antigens. Binding studies were performed with 28K and 18K versions of the superantigen and showed that both molecules bind to human MHC class II antigens (Mottershead et al., 1995). Thus, studies by our laboratory indicate one site for binding to mouse MHC class II antigens, whereas studies performed by others suggest that two sites on MMTV superantigen are involved in binding to human MHC class II antigens.

Despite the diverse origins of SEA and MMTV vSAg protein, it is likely that these two proteins exert superantigen activity by a similar mechanism. Two different regions of the MMTV superantigen are probably involved in MHC binding and Vβ specificity. The variability of the C-terminal 30 residues between Vβ-specific MMTV superantigens is indirect evidence that the C-terminus is responsible for Vβ specificity (Pullen et al., 1992). Data presented here on competitive and direct binding of vSAg peptides to A20 cells suggest that the region encompassed by residues 76-119 is involved in MHC binding.

This region would be part of the extracellular domain based on a proposed Type II membrane protein model for MMTV vSAg protein (Choi et al., 1992). Thus, both the C-terminal tail and an N-terminal region may be required for ternary complex formation with MHC and TCR by MMTV superantigen. Conversely, SEA contains several N-terminal domains that bind MHC (Griggs et al., 1992) and an internal domain that may be involved in binding to TCR, so that the sites of interaction of these superantigens with MHC and TCR may not be completely analogous. Although little sequence homology exists between MMTV vSAg protein and SEA, circular dichroism analysis indicates that both SEA and vSAg(76-119) have significant β structure, suggesting that similar structural motifs may be important for MHC binding by superantigens.

Finally, results presented herein indicate that a peptide corresponding to residues 76-119 of the MMTV superantigen binds directly to MHC class II antigens. Further, competition studies indicate that SEA and vSAg(76-119) peptide bind to at least one common region on mouse MHC antigens. Future studies using synthetic peptides and peptide analogues may help further elucidate the site(s) on MHC for which SEA and MMTV superantigen compete.

CHAPTER III
IDENTIFICATION OF AN HIV-1 NEF PEPTIDE THAT BINDS TO MHC CLASS II ANTIGENS

Introduction

In Chapter Two, a site was identified on the MMTV-1 superantigen that binds to class II MHC antigens, suggesting that retroviral and bacterial superantigens exert their effects similarly. MMTV superantigen is encoded in a gene that overlaps the terminal repeat at the 3' end of the viral genome. The genome of human spumavirus also contains a gene (*bel3*) that overlaps the terminal repeat at the 3' end, the product of which may have superantigenic properties (Fluegel, 1993). Recently, it has been suggested that human immunodeficiency virus (HIV) may possess superantigen activity, although no specific superantigenic protein has been identified (Imberti et al., 1991; Laurence et al., 1992; Dalgleish et al., 1992; Bisset et al., 1993). The HIV genome also contains a gene that overlaps the terminal repeat at the 3' end, the product of which is called Nef. Although Nef is one of the early proteins produced during the replication of primate lentiviruses, the role of Nef in HIV pathogenesis has yet to be established.

To determine if Nef had binding characteristics similar to superantigens, a study was undertaken to determine its ability to bind to MHC class II antigens, at sites that are known to bind superantigens. Using overlapping peptides corresponding to the entire length of Nef (HIV_{LAV}), we have identified a region which binds to MHC class II antigens, and which competes for binding with known bacterial superantigens.

Materials and Methods

Synthetic Peptides

Overlapping peptides corresponding to the entire length of HIV_{LAV} Nef (Wain-Hobson et al., 1985) were synthesized with a Biosearch 9500AT automated peptide synthesizer using N-(9-flurenyl)methoxycarbonyl chemistry as described in chapter II of this text. Peptides were synthesized based on a surface profile as described. The amino acid sequences of the peptides are presented in Table IV.

Cells and Reagents

Two cell lines were used for the binding studies. Raji cells are EBV-transformed B cells that express DR3, Dw10, DQw1, and DQw2 (Merryman et al., 1989). DR1-transfected L cells were kindly provided by Dr. Eric O. Long and are described elsewhere (Lechler et al., 1988). SEs were obtained from Toxin Technology (Sarasota, FL). Several mAb were used in this study. Anti-HLA-DR clone L243 reacts with a nonpolymorphic DR epitope and does not cross-react with DP or DQ (Robbins et al., 1987). Anti-HLA-DP clone B7/21 reacts with a monomorphic epitope present on DP1, DP2, DP3, DP4, and DP5 (Robbins et al., 1987). Anti-HLA-DQ clone SK10 reacts with a common polymorphic epitope present on cells expressing DQw1 and DQw3 (associated with DR1. DR2, DR4, DR5, w8, w9, and w10) (Brodsky, 1984). Anti-HLA-DR clone L227 reacts with a nonpolymorphic region of DR (Barnstable et al., 1978). Clone W6/32 reacts with a monomorphic epitope on HLA-A, B, and C (Barnstable et al., 1978). Clones L243, B7/21, and SK10 were obtained from Becton-Dickinson (Mountain View, CA) and clones L227 and W6/32 were kindly provided by Dr. Robert Rich. All mAbs were used at a final concentration

Table IV.
Amino acid sequences of Nef peptides.

Nef Peptide	Sequence
1-38	MGGKWSKSSVVGWPTVRERMRRAEPAADGVGAASRDLE
31-65	GAASRDLEKHGAITSSNTAATNAACAWLEAQEEEE
62-99	EEEEVGFPVTPQVPLRPMTYKAAVDLSHFLKEKGGLEG
93-132	EKGGLEGLIHSQRRQDILDLWIYHTQGYFPDWQNYTPGPG
123-160	DWQNYTPGPGVRYPLTFGWCYKLVPVEPDKVEEANKGE
156-186	NKGENTSLLHPVSLHGMDDPEREVLEWRFD
182-206	EWRFDSRLAFHHVARELHPEYFKNC

of 10 μg/ml. Purified recombinant Nef protein was purchased from Repligen, Cambridge, MA.

Radioiodinations

SEs (2.5 μg) and Nef peptide (10 μg) were radioiodinated using chloramine T as described in chapter II of this text. The specific activities of the SEs and Nef peptide ranged from 70-120 μCi/μg and 30-40 μCi/μg, respectively.

Binding Studies

For binding studies on Raji cells, unlabeled competitors (SEs and Nef peptides) were added in 50 μl (in PBS with 1% BSA) to 50 μl of cells in 1.5 ml "Eppendorf" tubes. Cells and competitors were incubated at room temperature for 45 min., followed by the addition of radiolabeled SEs or Nef peptide. After 45 min., the cells were washed three times and the bottoms of the tubes were cut off. Radioactivity was quantified using a gamma counter. Similarly, DR-1 transfected L cells, which were grown to confluence in the wells of microtiter plates, were subjected to the same incubation times and volumes of competitors and labeled ligands as used for the Raji cells. After three washes, the cells were solubilized in 1% SDS, and the liquid was absorbed by cotton-tipped applicators and radioactivity was quantified using a gamma counter.

Results

Competition of Nef Peptides with Radiolabeled SEA for MHC Binding

Overlapping peptides corresponding to the entire length of the Nef sequence (HIV_{LAV}) were synthesized (Table IV). The Nef peptides were initially tested at a concentration of 300 μM for their relative abilities to compete with ^{125}I-SEA for binding to Raji cells, which express HLA-DR3 and HLA-DRw10, and DR1-transfected L cells (Figure 6). Nef(123-160) reduced ^{125}I-SEA binding to Raji cells by 41% which was significant at $p<0.002$. This degree of inhibition was consistent in repeated experiments. The N-terminal peptide, Nef(1-38), had a marginal but insignificant effect (21% reduction, $p>0.05$) on ^{125}I-SEA binding to Raji cells in the same experiment. This slight inhibition by Nef(1-38) was not seen in repeated experiments. The other peptides did not affect SEA binding to Raji cells. A similar pattern was seen in competitive binding studies performed on DR1-transfected L cells. Nef(123-160) blocked ^{125}I-SEA binding to DR1 cells by 35% ($p<0.008$), whereas Nef(1-38) had only a slight effect ($p>0.8$). Thus, only one of the overlapping Nef peptides, Nef(123-160), significantly and consistently blocked ^{125}I-SEA binding to both Raji cells and DR1-transfected L cells.

Nef(123-160) Peptide Competition Is Dose-Dependent

The relative abilities of Nef(123-160), purified recombinant Nef protein (Repligen, Cambridge, MA), and SEA to block SEA binding to MHC class II were tested in dose response studies (Figure 7). Nef(123-160) reduced ^{125}I-SEA binding to Raji cells by 40% at the highest concentration tested (300 μM), and competed with SEA in a dose-dependent manner. Two other Nef peptides, Nef (1-38) and Nef(31-65), did not block SEA in a dose-dependent manner. Unlabeled SEA reduced ^{125}I-SEA binding by 50% at a concentration of 0.2 μM, which is consistent with the reported Kd of SEA for human MHC class II antigens (Chintagumpala et al., 1991). Thus, Nef protein and the internal Nef sequence, Nef(123-160), competed for SEA binding to Raji cells in a dose-dependent manner.

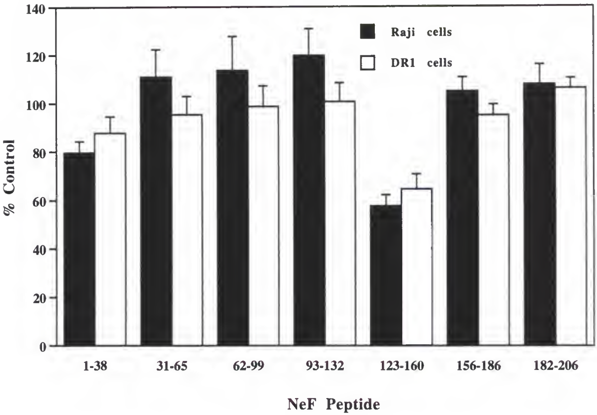

Figure 6. Blockage of ^{125}I-SEA binding to Raji and DR1-transfected L cells by Nef peptides. Nef peptides were used at a final concentration of 300 µM. ^{125}I-SEA was used at a final concentration of 2 nM. 10^5 Raji or DR1-transfected L cells were used per tube. Binding of ^{125}I-SEA to Raji and DR1-transfected L cells in the absence of competitors was 31,469 ± 2292 and 5,708 ± 41 CPM, respectively. Data represent the mean percent of control of three individual experiments, each performed in duplicate. Bars represent binding to Raji and DR1-transfected L cells in the presence of Nef peptides ± SD.

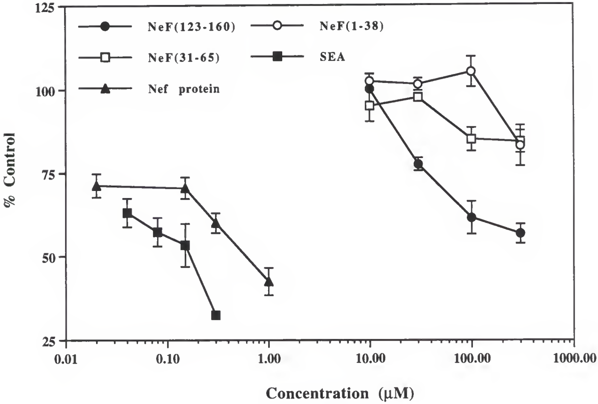

Figure 7. Relative abilities of Nef(123-160), purified Nef protein, and SEA to compete with ^{125}I-SEA for binding to Raji cells. Experimental conditions were the same as those described in Figure 1. Binding of ^{125}I-SEA to Raji cells in the absence of competitors was 30,406 ± 2851 CPM. The data presented represent the mean of three individual experiments, each performed in duplicate. Each point represents the mean percent of SEA control binding in the presence of competitors ± SD.

Ability of Nef Peptides to Compete with SEs for Binding to Raji Cells

Nef peptides were also tested for their relative abilities to compete with other staphylococcal enterotoxins (SEs) for binding to Raji cells. As shown in Figure 8, Nef(123-160) significantly blocked binding of ^{125}I-SEE by 42% ($p<0.02$) and ^{125}I-SEC1 by 26% ($p<0.03$), but only blocked binding of ^{125}I-SEB by 10% ($p>0.1$). Thus, Nef(123-160) significantly inhibited the binding of two highly homologous SEs, SEA and SEE, while it was less effective against SEB and SEC1.

Direct Binding of Nef(123-160) Peptide to Raji Cells

In direct binding studies, ^{125}I-Nef(123-160) bound to Raji cells and was effectively inhibited by unlabeled Nef(123-160) and SEs (Figure 9). Unlabeled Nef(123-160) reduced ^{125}I-Nef(123-160) binding by 50% at a concentration of 30 µM. SEE was a better competitor and reduced ^{125}I-Nef(123-160) binding by 50% at a concentration of 2 µM. Both SEE and unlabeled Nef(123-160) competed in a similar manner, but SEE was a more potent competitor. SEA did not inhibit ^{125}I-Nef(123-160) binding as well as SEE, but it was more effective than SEC_1 or SEB. This pattern of inhibition is reflective of the ability of Nef(123-160) to block SE binding to MHC class II antigens. Although SEs compete for similar sites on MHC class II antigens, our results suggest that Nef(123-160) competes for sites that are more closely associated with SEE than the other SEs tested. Nef(123-160) binding was not blocked by other Nef peptides, such as Nef(1-38) and Nef (31-65), at 300 µM. These results suggest that Nef(123-160) binds directly to Raji cells and competes for a site on MHC class II antigens to which SEs bind.

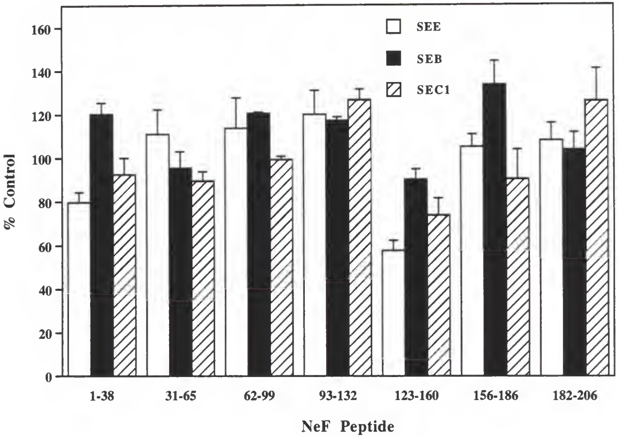

Figure 8. Blockage of ^{125}I-SE binding to Raji cells by Nef peptides. Nef peptides were used at a final concentration of 300 μM. ^{125}I-SEs were used at 2 nM. All other experimental conditions were the same as those described in Figure 7. Binding of ^{125}I-SEE, ^{125}I-SEB, and ^{125}I-SEC1 in the absence of competitor was 5,917±612 CPM, 6,438±134 CPM, and 4,493±425 CPM, respectively. Data represent the mean of three individual experiments, each performed in duplicate. Each bar represents the mean percent of the appropriate SE control binding to Raji cells in the presence of Nef peptides ± SD.

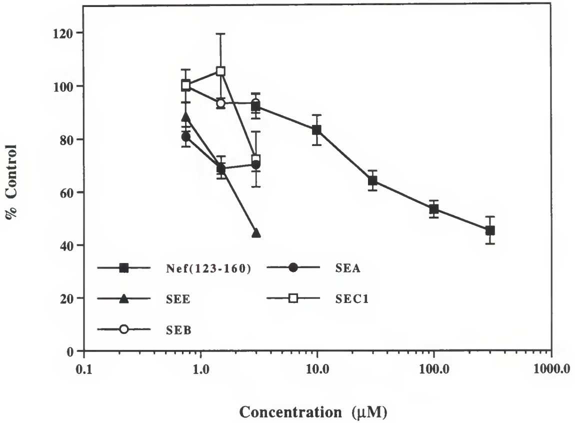

Figure 9. Direct binding of Nef(123-160) peptide to Raji cells. ^{125}I-Nef(123-160) was used at a final concentration of 3 nM. All experimental conditions were the same as those described in Figure 7. Binding of ^{125}I-Nef(123-160) in the absence of competitor was 1956 ± 150 CPM. The data presented represent the mean of three individual experiments, each performed in duplicate. Each point represents the mean percent of ^{125}I-Nef(123-160) binding relative to control in the presence of competitors ± SD.

MAbs to MHC Class II Antigens Block Nef(123-160) Peptide Binding to Raji Cells

To ascertain the receptor to which ^{125}I-Nef(123-160) bound on Raji cells, binding was performed in the presence of monoclonal antibodies (mAb) to class II and class I antigens (Figure 10). Clone L243, a mAb specific for HLA-DR, reduced ^{125}I-Nef(123-160) binding by 35%. Another mAb specific for HLA-DR, clone L227, also reduced Nef(123-160) binding, but was not as effective as clone L243. This is consistent with reports that L227 mAb is not very effective at blocking SEA binding to Raji cells (Chintagumpala et al., 1991). MAb to other class II antigens (HLA-DP and DQ) had no effect on ^{125}I-Nef(123-160) binding, as was also the case for a mAb to class I antigens (clone W6/32). Thus, our studies indicate that Nef(123-160) binds to class II MHC antigens, the known receptors for superantigens on APC.

Discussion

Data presented here show that binding of bacterial superantigens to MHC class II antigens can be blocked by a peptide corresponding to an internal Nef region. Anti-HLA-DR mAb blocked Nef(123-160) binding to Raji cells, suggesting that binding to class II molecules occurred. Binding of Nef(123-160) to HLA-DR probably occurs outside the antigen binding groove, since Nef(123-160) was able to block binding of the superantigens SEE and SEA. A recent study has shown that HLA-DR is present on HIV-1 and SIV, and that it is selectively incorporated into the virus membrane over HLA-DP or HLA-DQ (Arthur et al., 1992). Antibodies to HLA-DR, but not antibodies to HLA-DP or HLA-DQ, effectively inhibited viral infection of cells *in vitro* and these antibodies target the HLA-DR antigens present on the virus particles and not those present

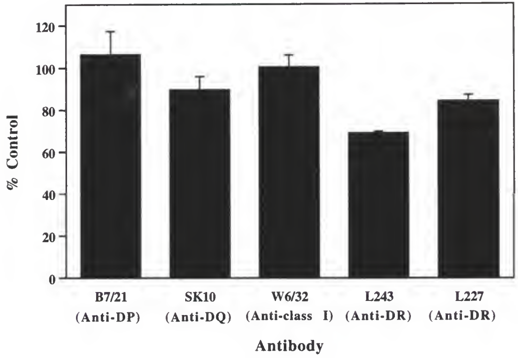

Figure 10. Blockage of ^{125}I-Nef(123-160) binding to Raji cells by antibodies to MHC class I and class II antigens. ^{125}I-Nef(123-160) was used at a final concentration of 3 nM. MAbs were used at a final concentration of 10 μg/ml.

on the cells. The ability of these antibodies to neutralize HIV suggest that HLA-DR may play a role in infectivity. Our results suggest that Nef may bind to HLA-DR as it is being expressed on the surface of an infected cell in a manner analogous to the putative expression of MMTV superantigen (Choi et al., 1992). Further, Nef has been shown to be present on the surface of infected cells, as assessed using anti-Nef antibodies labeled with fluorescein (Fujii et al., 1993). This is in contrast to the situation with MMTV superantigen in that no one has been able to show that cells from MMTV-infected animals express superantigen, although superantigen functional activity is observed (Choi et al., 1991). Transgenic mice expressing MMTV superantigen delete $V\beta14^+$ T cells, probably during the ontogeny of the immune system, and these mice are immune to infection by exogenous MMTV infection (Acha-Orbea and Palmer, 1991). Thus it is possible that Nef interacts with class II MHC antigens in a manner somewhat similar to MMTV superantigen, suggesting a possible superantigen function for Nef in HIV pathogenesis.

CHAPTER IV
ACTIVATION OF CD4 T CELLS BY THE NEF PROTEIN FROM HIV-1

Introduction

In the previous chapter, data was presented which showed that Nef binds specifically to class II-bearing Raji cells at a site(s) involved in staphylococcal enterotoxin binding. This binding was similar to that shown for MMTV superantigen. These data suggest that Nef may have characteristics of superantigens. The data presented in this chapter show that Nef induces proliferation of human peripheral blood mononuclear cells. Further, the proliferative response is T cell specific. The data presented in this chapter indicate that Nef activates T cells and induces T cell cytokine production in a manner reminiscent of staphylococcal superantigens.

Materials and Methods

Nef Protein

Nef protein was expressed and purified using a fusion protein and purification system. The HIV-1 nef gene (HIV-1 III$_B$, R & D Systems, Cambridge, MA) was amplified by polymerase chain reaction (PCR), using the following primer set :

5' ATG GGT GGC AAG TGG TCA AAA AGT (+)

5' GCC AAG CTT GAT GTC AGC AGT TCT (-)

The extra A added to the 3' end of the amplified *nef* gene fragment as a result of PCR was removed by treatment with the Klenow fragment of DNA polymerase. This DNA was ligated into the *Xmn*I/*Hin*dIII cloning site of the prokaryotic expression vector pMAL™-c2 (New England BioLabs, Beverley, MA). The sequence of the fusion construct was verified by the DNA Sequencing Laboratory, Interdisciplinary Center for Biotechnology Research, University of Florida. The fusion construct was engineered such that the N terminus of Nef was immediately downstream from the Factor Xa cleavage site at the C terminus of maltose binding protein (MBP). Thus, cleavage with Factor Xa was predicted not to add any vector-derived amino acids. *E. coli* strain TB1 was transformed with the vector containing *mbp/nef*. Cultured *E. coli* TB1 were suspended in column buffer consisting of 20 mM Tris-HCl (pH 7.4), 200 mM NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA). The cells were sonicated and the supernatants were recovered after centrifugation. Protease inhibitors (2 mM phenylmethylsulfonyl fluoride (PMSF), pepstatin A, leupeptin, and aprotinin) were added to extracts. The protein concentration of the extracts was adjusted to 2.5 mg protein/ml prior to loading onto an amylose affinity column containing a bed volume of 50 ml. The column was washed with 350 ml of column buffer. The fusion protein was eluted with 200 ml of column buffer containing 10 mM maltose. Fractions (7 ml) were collected. The fusion protein generally eluted within the first 70 ml. After cleaving the fusion protein with factor Xa (New England BioLabs, Beverley, MA), the cleavage mixture was loaded onto a hydroxyapatite column containing a bed volume of 35 ml. After extensive washing of the column with 180 ml of 10 mM potassium phosphate (pH 7.2) containing 2 mM PMSF and 1 mM benzamidine, fractions were eluted using a linear gradient from 10 mM to 400 mM potassium phosphate buffer (pH 7.2) containing 2 mM PMSF. Fractions (5 ml) were collected. Nef eluted from the column between 100-150 mM potassium phosphate, and was generally contained within 7 fractions. The fraction containing Nef protein was dialyzed against affinity column buffer containing 150 mM NaCl. The dialyzed material was loaded onto a second amylose affinity column for removal of MBP. The flow-through containing Nef protein was collected and loaded onto an High-Q ion-exchange cartridge (BioRad Laboratories, Richmond, CA). The High-Q cartridge was washed with 100 ml of 20 mM Tris buffer (pH 8.0) containing 150 mM NaCl. Fractions were eluted with a linear gradient from 150-400 mM NaCl in the same buffer. The fractions containing Nef protein, which eluted at 180 mM NaCl, were collected and dialyzed overnight against PBS and stored at -70°C. The purity of Nef protein was assessed by SDS-PAGE, followed by staining with silver. Upon staining with silver, a single band was found, and a corresponding band was detected by Western blotting with monoclonal anti-Nef antibody (Repligen, Cambridge, MA). MBP purified by this method and used at the same concentrations as that used for the purified Nef preparation, did not have proliferative activity on human peripheral blood mononuclear cells.

Nef proliferative activity was confirmed using recombinant Nef proteins obtained from two other sources and which were produced using different expression systems. The following reagent was obtained through the AIDS Research and Reference Reagent Program, AIDS Program, NIAID, NIH: HIV-1 LAV Nef from the Division of AIDS, NIAID. The HIV-1 nef gene (LAV) used to produce this Nef preparation was isolated from pBENN 6 and cloned into the bacterial expression vector pPD-YN-61. The protein was produced in *E. coli* strain Sf930 and isolated as inclusion bodies. Nef protein was also obtained from Repligen (Cambridge, MA). In this case, the HIV-1 nef gene (LAV) was cloned into the bacterial vector pD10, which adds a hexahistidine tag for protein purification using nickel chelate chromatography. The protein was produced in

E. coli strain MC10611POL.1. All Nef preparations were negative for endotoxin as assessed by the limulus amoebocyte lysate assay.

Reagents

Staphylococcal enterotoxin A (SEA) was purchased from Toxin Technology (Sarasota, FL). Concanavalin A (Con A), anti-CD3, and anti-IgM were purchased from Sigma Chemical Co. (St. Louis, MO).

Proliferation Assays

Proliferation studies on human peripheral blood mononuclear cells (PBMC) were performed as described previously (Pontzer et al., 1992). Peripheral blood from healthy adult blood bank donors was obtained from Civitan Regional Blood Center (Gainesville, FL) for use in these studies. All donors were negative for cytomegalovirus, hepatitis B virus, and HIV. PBMC were isolated from peripheral blood using ficoll hypaque gradient centrifugation. After extensive washing, PBMC were plated into wells of microtiter plates at 2.8 x 10^6 cells/ml, followed by addition of activators. Final volumes were adjusted to 150 µl/well with RPMI 1640 tissue culture medium containing 5% fetal bovine serum (FBS) and penicillin/streptomycin. ^{3}H-thymidine (1µCi/well; Amersham Corporation, Indianapolis, IN) was added at 90 h after initiation of cultures and the cells were incubated for an additional 6 h prior to harvest onto filter paper. Filter paper was placed in liquid scintillation fluid and radioactivity was quantified using a β scintillation counter. Stimulation index (S.I.) was determined by dividing the experimental CPM by the CPM obtained from control (unstimulated) cultures.

Purification of T Cells from PBMC

Two methods were used to isolate purified T cell populations. One method involved passing PBMC over T Cellect columns (Biotex, Edmunton, Alberta, Canada). A second method involved a double rosetting technique using neuraminidase-treated sheep red blood cells (NA-SRBC) (Spawski and Lipsky, 1992). PBMC (5 ml at 10^7/ml) were mixed with 2.5 ml each of NA-SRBC and FBS. PBMC were incubated for 10 min at 37°C, centrifuged at 900 rpm to gently pellet the cells, and incubated for 90 min at 37°C. Rosetted T cells were separated from non-rosetted cells by ficoll hypaque centrifugation. NA-SRBC were removed from the rosetted fraction by lysis with ammonium chloride. The procedure was repeated on the rosetted fraction to insure purity. Autologous rosetted T cells were plated at 2.8×10^6 cells/ml. Cells were plated into wells of microtiter plates at 2.8×10^6 cells/ml. To insure that these cultures were depleted of APC, T cells were stimulated with staphylococcal enterotoxins, which stimulate T cells only in the presence of APC.

Autologous Antigen-Presenting Cells (APC)

For proliferation studies, APC consisted of PBMC that were inactivated with 0.8% paraformaldehyde. For cytokine studies, APC consisted of cells that did not form rosettes after two treatments with NA-SRBC. APC were plated at 2.8×10^6 cells/ml. To insure inactivation with paraformaldehyde was complete, APC were tested for possible proliferation using anti-IgM and Con A.

Synthetic Nef Peptides and Antibodies to Nef Peptides

The sequences of the peptides used are listed in Table III. Peptides were purified by reverse phase high performance liquid chromatography. Polyclonal antibodies to Nef peptides were generated in rabbits. Anti-peptide antibodies were tested for their relative abilities to neutralize Nef mitogenic activity by incubating Nef with antibodies at 37°C for 60 minutes prior to addition of PBMC. Antibodies to both Nef(123-160) and Nef(182-206) showed strong reactivity to whole recombinant Nef protein, and these combined antisera had significant neutralizing activity against Nef. Nef was treated with preimmune sera as control.

Cytokine Production and Assays

PBMC were plated at 10^6 cell/ml into wells of 24-well plates (Corning Corp., Corning, NY). Activators were added and final volumes were adjusted to 300 µl/well. Cultures were incubated at 37°C over a 96 h period and samples (100 µl) were obtained at 24 h intervals. Cell cultures were replenished with RPMI/5% FBS (100 µl) after obtaining samples. Culture supernatants were tested for interleukin 2 (IL 2) using the IL 2-dependent HT-2 cell line (Ho et al., 1987). The amounts of IL 2 present in culture supernatants were determined using recombinant human IL 2 (Genzyme, Cambridge, MA) as a standard. Samples were tested for interferon (IFN) activity on human WISH cells by a microplaque reduction method (Langford, et al., 1978b), using approximately 40 plaque-forming units (PFU) of vesicular stomatitis virus (VSV) per well. In our studies, 1 U/ml of IFN is defined as the concentration required to decrease the number of PFU per well by 50%.

IFN activity was typed by neutralization reactions with specific antisera, as described (Johnson et al., 1982). Briefly, samples were pretreated with 1000 neutralizing units of either anti-human IFNα (Lee Biomolecular, San Diego, CA) or anti-human IFNγ (Genzyme, Cambridge, MA). Controls were sham-treated with EMEM/2% FBS. The neutralizing activity of anti-IFN antisera was confirmed using HuIFNα (Lee Biomolecular, San Diego, CA) and HuIFNγ

(Genzyme, Cambridge, MA) as positive controls. Samples were incubated at 37°C for 1 h prior to transfer to confluent human WISH cells. Residual IFN activity in sham- and antiserum-treated samples was measured as described above.

Studies on Vβ-Specific T Cell Expansion and Induction of Anergy

PBMC cultured in the presence of Nef, TSST-1, and ConA were tested by flow cytometry for specific Vβ expansion as previously described (Soos and Johnson, 1994) using a panel of anti-Vβ antibodies. Induction of anergy *in vitro* by Nef, TSST-1, and ConA was also assessed with anti-Vβ antibodies as described (Schiffenbauer et al., 1993). Antibodies to Vβ5a, Vβ5b, Vβ5c, Vβ6a, Vβ8a, and Vβ12 were obtained from T Cell Sciences, Inc., Cambridge, MA. Antibodies to Vβ2, Vβ3, Vβ13, Vβ17, Vβ18, Vβ21, and Vβ22 were purchased from Immunotech, Marseilles, France.

Results

Nef Proliferative Response

Recombinant Nef proteins from two sources were tested for proliferative activity. One Nef preparation (referred to as Nef 1) was purified and kindly provided by Dr. Taishi Tanabe in the laboratory of Dr. Howard M. Johnson. This Nef preparation was compared to purified Nef from Repligen (Nef 2). Both Nef protein preparations, which are derived from the HIV_{LAV} sequence, induced similar levels of proliferation (Figure 11). Nef proteins from both sources were pure and neither preparation contained detectable levels of endotoxin. Thus, Nef proteins from both sources had similar proliferative activity, and the proliferation observed was significant. Nef from these two sources were

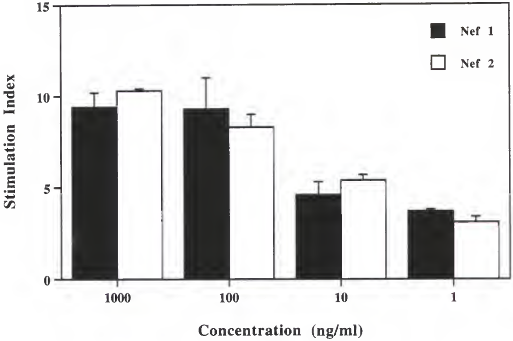

Figure 11. Comparison of the mitogenic activities of Nef protein preparations. Two Nef preparations,Nef 1 and Nef 2, were compared for proliferative activities on human PBMC. Data are from a representative experiment, performed in triplicate, and are expressed as mean stimulation index ± S.D.. Comparison of the proliferation of PBMC cultured for 4 days in the presence of either Nef 1 or Nef 2. The mean value for 3H-thymidine incorporation by unstimulated cultures was 900±67.

subsequently used in these studies. The possibility was raised that the proliferative activity ascribed to Nef was due to a contaminant in the recombinant preparation. As a control, the proliferative activity of the Nef fusion partner, MBP, was also assessed. No proliferation was observed with MBP (Figure 12). Further, polyclonal antisera generated against Nef synthetic peptides were tested for neutralizing activity. As shown in Table V, anti-Nef antibodies significantly reduced Nef-induced proliferative responses. Antibody neutralization of Nef activity occurred in a dose-dependent manner. These data confirm that the proliferative activity seen in these studies is due to Nef protein, and not to a vector-derived contaminant.

Nef was also tested for the ability to induce proliferation of PBMC from fifteen donors. Results from a representative sample of ten donors are shown in Figure 13. Nef induced significant proliferation in 90% of the donors tested. Variation in the Nef response was seen, similar to the responses to the potent staphylococcal enterotoxin, SEA. It is unlikely that these donors were sensitized to Nef, since they tested negative for HIV-1. Similar results were obtained using purified recombinant Nef protein preparations from two different sources, and which used different expression systems (see Materials and Methods in this chapter). The high number of donors that responded to Nef indicates Nef protein has similar mitogenic activity to SEA for PBMC from a wide sampling of donors.

Antigen-Presenting Cells (APC) Are Required for Nef Activity

The question arose as to whether Nef, like SEA, requires APC in order to stimulate lymphocyte proliferation. Nef induced significant proliferation in PBMC (Figure 14). Purified T cell cultures did not proliferate in response to Nef or to SEA. Purified APC, which contained monocytes and B cells, did not

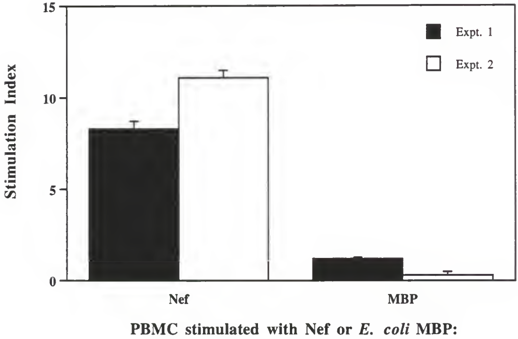

Figure 12. Lack of proliferation by PBMC in response to the Nef fusion partner MBP. MBP and Nef were used at 3 µg/ml. Data are expressed as mean stimulation index ± SD. The mean values for ^{3}H-thymidine incorporation by unstimulated cultures in eperiments 1 and 2 were 852±39 CPM and 1093±136 CPM, respectively.

Table V.
Ability of anti-peptide antibodies to block Nef-induced proliferation

PBMC cultured in the presence of[a]:	Anti-Nef antibodies[b]	Stimulation Index[c] (Mean ± SD)	p value
Nef	-	4.7 ± 0.2	
Nef	+	1.3 ± 0.1	<0.001
SEA	-	59.7 ± 2.6	
SEA	+	62.0 ± 0.6	>0.15
-	+	0.9 ± 0.4	
-	-	-	

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[a] Nef (3 µg/ml) and SEA (0.3 µg/ml) were incubated with anti-Nef antibodies for 60 minutes prior to addition of PBMC.

[b] A mixture of antisera to Nef(123-160) peptide and Nef(182-206) peptide were used, each at a final dilution of 1:1000. Preimmune sera had no effect. Both anti-Nef(123-160) and anti-Nef(182-206) had strong reactivity to Nef protein by ELISA.

[c] Data are from a representative experiment performed in triplicate.

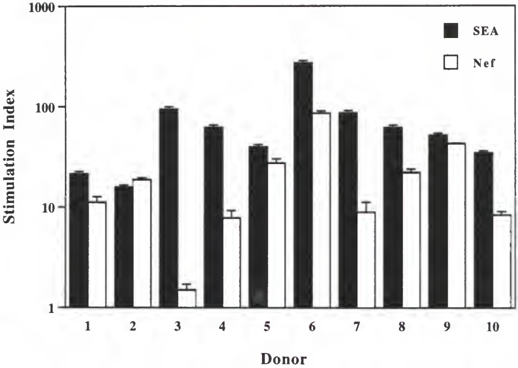

Figure 13. Nef-induced proliferative responses from a representative sampling of donors. PBMC of blood bank donors were tested for proliferation induced by SEA or Nef. SEA and Nef were used at 300 ng/ml. Mean values for ^{3}H-thymidine incorporation by unstimulated cultures ranged from 352±86 CPM to 1983±274 CPM.

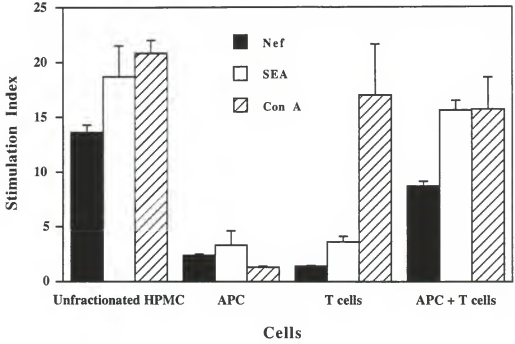

Figure 14. Nef-induced activation of T cells requires APC but does not require processing of Nef. Unfractionated PBMC, purified APC alone, purified T cells alone, and purified T cells in the presence of APC were tested for proliferation in response to Nef, SEA, and Con A. Data are from a representative experiment, performed in triplicate, and are expressed as mean stimulation index ± S.D. Purified T cells were reconstituted with paraformaldehyde-inactivated APC. Con A was used at 10 µg/ml. Nef and SEA were both used at 100 ng/ml.

respond to stimulation by either Nef or SEA. Upon reconstitution with paraformaldehyde-inactivated autologous APC, significant Nef-induced proliferation of T cells occurred, with essentially complete reconstitution of the response. Similar proliferative responses were obtained using cells from other donors. Thus, T cells responded to Nef stimulation only in the presence of autologous APC, and Nef does not require processing to be presented by APC. These results indicate that Nef-induced proliferation required APC in a manner reminiscent of SEA.

Nef(123-160) Peptide Specifically Blocks Proliferation of PBMC Induced by Nef and SEA

As shown in Chapter 3, a synthetic peptide corresponding to an internal Nef sequence, Nef(123-160), blocked binding of Nef and SEA to Raji cells. It was important to determine if this peptide could also block proliferation induced by Nef and SEA. The results of this study are presented in Figure 15. Nef(123-160) blocked both Nef-induced and SEA induced proliferation, consistent with its ability to block binding of Nef and SEA to Raji cells. Further, the blocking was specific in that proliferation induced by the T cell mitogens Con A and anti-CD3, or the B cell mitogen anti-IgM, were not blocked by Nef(123-160). Nef(157-186), whose sequence slightly overlaps that of Nef(123-160), had no effect on the proliferative effects of either Nef, SEA, or Con A. These results confirm that the proliferative responses observed in PBMC cultures were specific for Nef, and were not due to a contaminant. Thus, proliferative responses to Nef and SEA were specifically blocked by a peptide corresponding to the region on Nef that binds to class II antigens and which blocks binding of Nef and the superantigen SEA.

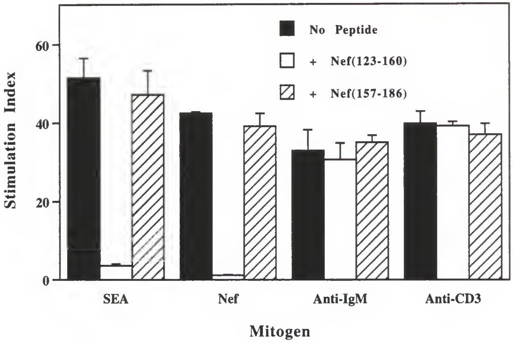

Figure 15. Nef(123-160) peptide specifically blocks proliferation of PBMC induced by Nef and SEA. PBMC cultures were stimulated for 96 h with mitogens in the absence of peptides or in the presence of either Nef(123-160) peptide or Nef(157-186) peptide. Data are from a representative experiment, performed in triplicate, and are expressed as mean stimulation index ± SD. Nef and SEA were used at 300 ng/ml. Anti-IgM and anti-CD3 were used at 10 μg/ml. Peptides were used at a final concentration of 100 μM. The mean value for ^{3}H-thymidine incorporation by unstimulated cultures was 1005±37 CPM.

Induction of T Cell Cytokines by Nef

T cell activation by the staphylococcal superantigens results in the prodigious production of T cell cytokines, such as IL 2 and IFNγ. Because of the significant T cell proliferation induced by Nef, it was determined if Nef induced Thelper cell cytokines. The results of a representative experiment are presented in Table VI. Consistent with proliferation data, Nef induced significant levels of IL 2, although IL 2 levels were lower than those induced by SEA. The question arose as to the ability of Nef to also induce another important T helper cell cytokine, IFNγ. Samples were removed from cultures of cells over the course of 96 h and tested for IFN antiviral activity. Nef induced high levels of IFN activity, with peak production occurring by 96 h (Figure 16, Panel A). IFN levels were lower than those induced by SEA, but were similar than those induced by Con A. Purified T cells did not produce IFN upon stimulation with either Nef or SEA (Figure 16, Panel B). However, purified T cell cultures were capable of producing IFN upon Con A stimulation. No IFN activity was produced in cultures of APC (consisting of B cells) stimulated with either Nef, SEA, or Con A. Purified T cell cultures reconstituted with purified B cells for antigen presentation produced significant levels of IFN upon either Nef or SEA stimulation (Figure 16, Panel C). These results are consistent with T cell mitogen activity for both Nef and SEA.

The type of IFN activity induced by Nef was determined by neutralization reactions with specific antisera. Treatment of Nef-induced IFN with anti-IFNγ resulted in a significant reduction of activity (Table VII). Antisera to IFNα did not affect the IFN activity in these samples. Consistent with previous data (Langford et al., 1978a; Johnson et al., 1982), the IFN activity induced by SEA was IFNγ. Similar to the results found with unfractionated PBMC, the IFN produced

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Table VI.
IL 2 production induced by Nef.

	IL 2 (U/ml) produced at[b]:	
Mitogen[a]	48 h	96 h
Nef	14.7 ± 0.1	16.9 ± 1.1
SEA	17.2 ± 0.7	24.7 ± 1.8
None	< 3	< 3

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[a] Samples were obtained from PBMC cultures which had been stimulated with Nef and SEA at 300 ng/ml and 500 ng/ml, respectively.

[b] IL 2 in culture samples were determined using IL 2-dependent HT-2 cells. The data presented are from a representative experiment performed in triplicate.

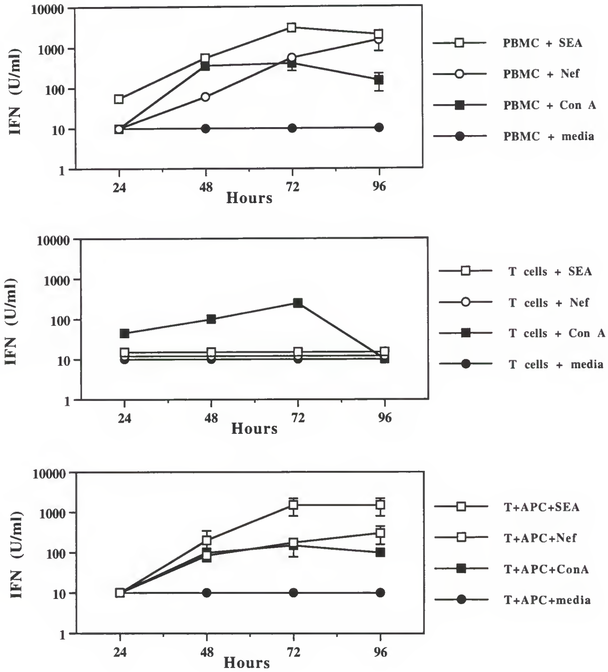

Figure 16. Kinetics of IFN production induced by Nef. Cultures of PBMC (Panel A), purified T cells (Panel B), and reconstituted cultures of purified APC and purified T cells (Panel C) were tested for IFN production at 24, 48, 72, and 96 h. Cultures were stimulated with either Nef at 100 ng/ml, SEA at 100 ng/ml, or Con A at 10 µg/ml, or were left unstimulated. Cultures of purified APC produced <10 U/ml IFN at all timepoints.

Table VII.
IFNγ is induced by Nef.

Samples from PBMC cultures stimulated with:	IFN titer (U/ml) after treatment with:		
	EMEM	anti-IFNα	anti-IFNγ
Nef	300	300	20
SEA	300	300	30
Con A	300	300	100
IFNα Control	300	<3	300
IFNγ Control	30	30	<3

by T cell cultures reconstituted with B cells in response to Nef was IFNγ. Thus, Nef induced high levels of IFNγ in cultures of PBMC, indicating that Nef activates T cells to produce the cytokines IL 2 and IFNγ, lymphokines that are products of activated T helper 1 cells.

PBMC cultured in the presence of Nef were tested for specific Vβ expansion and anergy *in vitro*. No expansion of specific Vβ populations was observed with the anti-Vβ antibodies used (data not shown). Unlike pretreatment with TSST-1, which anergized the response to anti-Vβ2, Nef did not significantly anergize the responses of T cells to the tested anti-Vβ antibodies (data not shown). Thus, no specific Vβ expansion or anergy was detected using the available human Vβ reagents.

Discussion

Nef protein induced significant levels of proliferation in unfractionated PBMC from a number of donors. The observed Nef responses were significant, with proliferation induced in cells from a large majority (85-90%) of the donors tested. It is unlikely that prior sensitization to Nef is responsible for the proliferative response, since these donors were negative for HIV. The following evidence points to Nef as the inducer of the observed proliferation: 1) proliferation was observed using recombinant Nef from three different expression systems; 2) antibodies to synthetic Nef peptides neutralized the proliferative activity of Nef; 3) the Nef fusion partner, MBP, did not induce proliferation; and 4) a synthetic peptide, Nef(123-160), previously shown to block binding of superantigens to MHC class II, blocked the Nef-induced proliferative responses. Nef induced proliferation was generally lower than that induced by SEA. This is not unusual in that SEA is extremely potent, causing proliferation at concentrations as low as 10^{-16} M (Langford et al., 1978a).

Proliferation induced by Nef occurred only in the presence of APC. Cultures of purified APC or purified T cells did not respond to Nef. T cells cultured in the presence of inactivated autologous APC proliferated to a similar extent to that seen in unfractionated cultures. These data suggest that Nef is presented to T cells by APC in an unprocessed form. The production of the T helper 1 lymphokines IL 2 and IFNγ from Nef-induced cultures is evidence that Nef activates T helper 1 cells.

In Chapter III, data was presented showing that Nef binds to Raji cells at a site(s) involved in SEA binding. A peptide corresponding to an internal region of Nef, Nef(123-160), specifically bound to DR 1-transfected L cells and blocked proliferation induced by Nef and SEA, evidence that Nef binds to MHC class II antigens. Con A-induced proliferation was not affected by Nef(123-160). This region of Nef was shown to be involved in binding of Nef to Raji cells. These functional data suggest that binding to MHC class II antigens is required for Nef proliferative activity. Binding of Nef to MHC class II occurs on APC, since Nef proliferative activity also requires presentation, but not processing, by APC. However, these data do not preclude Nef presentation by T cells expressing HLA-DR, a T cell activation marker.

Superantigenic activity of Nef was not observed using the anti-Vβ mAb currently available. Neither Vβ-specific expansion or anergy were observed. Further, preliminary results using reverse transcriptase-polymerase chain reaction (RT-PCR), which quantifies the amount of mRNA produced, did not show a Vβ preference in Nef-activated cells. These results raise a question on the nature of Nef receptor on T cells, and it is tempting to speculate on possible candidates such as TCR or CD4.

Nef induces human B cells to differentiate into immunoglobulin secreting cells (Chirmule et al., 1994). HLA-DR and adhesion molecules seem to be involved in Nef-induced B cell differentiation, since monoclonal antibodies to these surface proteins abrogated Nef-induced responses. Consistent with the findings on T cell activation by Nef, B cell differentiation induced by Nef required T cells. In addition to activating T cells, staphylococcal superantigens have also been shown to induce B cell differentiation (Stohl et al., 1994). Thus, additional parallels can be drawn between the functional activities of staphylococcal superantigens and Nef.

It has been shown that HIV requires activated T cells in which to replicate. Specific antiviral immune responses may not be sufficient to activate large numbers of T cells. For this reason, the identification of an HIV protein that induces T cell proliferation is of considerable interest and may, in part, explain the role of Nef in HIV pathogenesis. Activation of T cells may result from interaction with Nef, either in soluble form released from lysed infected cells or as a cell-associated complex with HLA-DR on the surface of infected T cells. T cell activation by Nef could result in a stable cellular reservoir for virus production as a result of continuous stimulation. In fact, hyperimmunization against Nef has been proposed as a means of reducing viral load, either prophylactically or therapeutically (Montagnier, 1995).

The T cell expansion observed in response to Nef may not be the only mechanism of polyclonal activation of $CD4^+$ T cells for HIV replication. Recent evidence points to a superantigen encoded by the human herpesvirus, cytomegalovirus (CMV), that expands Vβ12-bearing T cells, thereby enhancing HIV replication in CMV-infected individuals (Dobrescu et al., 1995). It is not surprising that no expansion of Vβ12-bearing T cells was observed in response to Nef, since the donors used in the studies discussed in this chapter were negative for CMV. This versatility in polyclonal $CD4^+$ T cell expansion via the endogenous mitogen Nef and exogenous superantigens such as that of CMV probably play an important role in HIV pathogenesis. Clearly, the control of the mitogenic activity of these substances should help reduce the viral load in HIV-infected individuals.

CHAPTER V
HIV ENCODES FOR ITS OWN CD4 T CELL MITOGEN

Introduction

Nef protein is encoded in the genomes of both HIV-1 and the related primate virus simian immunodeficiency virus (SIV). The function that Nef plays in the pathogenesis of these viruses is uncertain, although its importance is reflected in studies in which a nef-deleted SIV mutant did not cause disease and protected against infection with the pathogenic wildtype strain (Daniel et al., 1992). Further, the viral load in SIV mutant-infected animals was considerably lower than in animals infected with the wildtype strain. However, challenge of neonatal macaques with the same attenuated SIV strain caused disease and, in some cases, death (Baba et al., 1995). The different outcomes of these two studies may involve differences in the immune system development between neonates and adults.

In Chapter IV, data was presented on the ability of Nef protein to induce proliferation of human peripheral blood mononuclear cells (PBMC) from a wide sampling of HIV-negative donors. Proliferative responses were T cell specific and were accompanied by production of cytokines such as interleukin 2 (IL 2) and gamma interferon (IFNγ), indicative of CD4 T cell activation. Nef-induced T cell proliferation and activation required the presence of antigen-presenting cells. These results are interesting in that T cell activation has been shown to be required for active HIV replication. Further, although resting or quiescent T cells can be infected, evidence suggests that HIV replication only occurs upon subsequent cellular activation. Herein, work is described that indicates that Nef activation of T cells is sufficient for HIV replication. These results show that Nef is an HIV-encoded mitogen that helps, at least in part, in establishing a cellular reservoir for virus replication.

Materials and Methods

PBMC Cultures for HIV Infectivity Studies

PBMC were isolated from peripheral blood of healthy adult donors as described in Chapter IV. PBMC were resuspended in RPMI 1640 medium containing 5% FBS and cultured in 25 cm^2 flasks (Sarstedt Inc., Newton, NC) at a concentration of 2×10^6 cells/ml in a final volume of 4 ml (or 8×10^6 cells/flask). Mitogens were added at the initiation of cultures. Nef, SEA, and PHA were used at 3 µg/ml, 0.1 µg/ml, and 10 µg/ml, respectively. After four days, PBMC were washed extensively in PBS, resuspended in RPMI 1640 medium containing 10% FBS, 10^{-6} M 2-mercaptoethanol, and 10 U rHuIL 2/ml. IL 2 is rapidly depleted in these cultures, and thus rHuIL 2 was added to medium. Cells were counted and all cultures were adjusted to 8×10^6 cells/flask. Cultures were infected with HIV at a final reverse transcriptase titer of 20,000 CPM/ml. Culture supernatants were harvested and cells were fed with fresh medium every third day for 12 days.

PBMC Cultures for Proliferation Assays

Concomitant with infectivity studies, PBMC were cultured in microtiter plates to monitor proliferation in response to Nef and mitogens. The culture conditions were the same as those described in Chapter IV. Nef and mitogens were used at the same concentrations as described above. Stimulation indices were calculated as described in Chapter IV.

Reverse Transcriptase (RT) Assay

Harvested culture supernatants (1 ml) were placed in 1.5 ml "Eppendorf" tubes and centrifuged at 17,000 rpm for 70 minutes. A cocktail containing Mg^{++} as the divalent cation, poly(rA)oligo(dT_{12-18}) as template primer, and 2.5 µCi of (^3H)TTP per sample was used to resupend the virus. Tubes were incubated at 37°C for 60 minutes, after which time samples were blotted onto filter paper discs. Discs were allowed to air-dry and were washed extensively in sequential baths. Specifically, discs were washed twice in 10% trichloroacetic acid (TCA) for 15 and 5 minutes, respectively. Discs were then placed in 5% TCA containing 0.1% SDS for 5 minutes. Finally, the discs were washed in 95% ethanol, after which they were allowed to air-dry. Discs were placed in vials containing scintillation fluid and radioactivity was quantified on a β-scintillation counter.

ELISA for HIV p24 Antigen

Levels of p24 antigen in infected culture supernatants were assessed using a sandwich ELISA (DuPont, Boston, MA). Levels of p24 (in ng/ml) were quantified according to the Manufacturer's recommendations based on a standard curve using purified p24 antigen (provided by Manufacturer).

Assay for Infectious Virus from PBMC Cultures

Supernatants from PBMC cultures infected for 6 days with HIV were used to assess the titer of infectious virus produced in these cultures. Supernatants were adjusted to achieve RT titers of 12,000 CPM/ml and added to SEA-stimulated PBMC cultures. Culture conditions were the same as those described above. RT activity at Day 9 of cultures was assessed as described above.

Fluorescent Antibody Cell Sorter (FACS) Analysis of Nef-Activated Cells

PBMC were stimulated for 4 days with either Nef (3µg/ml) or SEA (0.1 µg/ml). PBMC were washed and resuspended in FACS buffer containing 0.5% BSA and 10 mM sodium azide. Cells were incubated with fluorescein-labeled mAb to HLA-DR for 45 minutes and washed with FACS buffer. Cells were then incubated with phycoerythrin-labeled mAbs to either CD4 or IL 2 receptor (IL 2R) for 45 minutes. Cells were washed and analyzed on a FACScan (Becton-Dickinson, Mountain View, CA) at 10,000 events/sample. All mAbs used were obtained from Becton-Dickinson, San Jose, CA.

Studies on Proliferation of PBMC Induced by Autologous HIV-Infected Cells

PBMC cultures were stimulated as described above. PBMC were infected with HIV for 6 days, at which time the cells were washed and inactivated by overnight treatment with 2% paraformaldehyde. Inactivated HIV-infected cells were washed extensively to remove excess paraformaldehyde. Fresh autologous PBMC were cultured in microtiter plates in the presence of inactivated HIV-infected cells at a ratio of 3:1. After 4 days, ^{3}H-thymidine incorporation was assessed as described above.

Anti-Nef Peptide Antibodies

A mixture of antibodies to Nef(123-160) peptide and Nef(182-206) peptide were used to block proliferation in response to autologous HIV-infected cells. Antibodies were each used at a final dilution of 1:1000, as described in Chapter IV. Inactivated HIV-infected cells were treated with antibodies for 60 minutes prior to addition of fresh autologous PBMC. Preimmune sera were used and had no effect on proliferation induced by HIV-infected cells.

Results

HIV Replication in Nef-Stimulated PBMC Cultures

Since T cell activation occurs with exogenous Nef protein, the question arose as to whether Nef stimulation of PBMC was sufficient to induce HIV-1 replication. To this end, PBMC of several HIV-negative donors were tested for proliferation induced by Nef and other T cell mitogens (Table VIII). Consistent with previous data, PBMC from most of the donors proliferated significantly in response to Nef protein, as well as to the T cell mitogens SEA and phytohemagglutinin A (PHA).

Unstimulated and mitogen-stimulated PBMC were infected with HIV-1 and cultures were monitored over a 12-day period for signs of viral replication. Supernatants from unstimulated PBMC showed only marginal reverse transcriptase (RT) activity and p24 antigen production (Table VIII). RT and p24 antigen levels were high in cells stimulated with T cell mitogens, in particular SEA. Interestingly, Nef-stimulated cells produced moderate to high levels of RT and p24 antigen, indicating that these cells were capable of sustaining virus replication. In an attempt to quantify the number of infected cells in these cultures, PCR analysis of serially-diluted DNA was performed using nested primers for the *gag* gene. Results indicated 10- to 30-fold increases in the number of cells harboring virus in Nef-stimulated cultures, as compared to unstimulated controls. These data indicate that Nef stimulation resulted in a large number of infected cells and a concomitant increase in viral load.

Infectious Virus Is Produced by Nef-Stimulated Cultures

As a means of determining whether the virus produced by Nef-stimulated was infective, supernatants from HIV-infected cultures were tested for reinfectivity. SEA-stimulated PBMC were infected with virus from 6-day

Table VIII
Maximal proliferation, RT, and p24 antigen values from stimulated cultures

Donor	Stimulation	Maximal Proliferation		Maximal RT value		Maximal p24 antigen value	
		Mean ± SD	Fold increase	Mean ± SD	Fold increase	Mean ± SD	Fold increase
204	Nef	8108 ± 238	8.0	19014 ± 7141	10.9	256 ± 24	10240
	SEA	43684 ± 4316	43.3	29680 ± 3509	17.1	1024 ± 402	40960
	PHA	54874 ± 4711	54.4	35817 ± 5233	20.6	1814 ± 80	72560
	None	1009 ± 199	-	1740 ± 41	-	0.025 ± 0.01	-
205	Nef	12363 ± 864	10.1	26614 ± 3119	7.1	73 ± 2	1197
	SEA	73243 ± 10333	60.6	125515 ± 16634	33.5	1523 ± 332	24967
	PHA	57267 ± 455	47.6	91313 ± 11325	24.4	1668 ± 49	27344
	None	1228 ± 583	-	3732 ± 947	-	0.061 ± 0.052	-
206	Nef	18753 ± 1711	15.3	9823 ± 3709	18.8	30.6 ± 0.7	124
	SEA	124742 ± 10411	96.0	218412 ±49382	412.9	819 ± 153	3316
	PHA	N.D.	-	12006 ± 4132	22.7	38.1 ± 6.7	154
	None	1313 ± 356	-	529 ± 35	-	0.247±0.03	-

Table VII --continued

Donor	Stimulation	Maximal Proliferation		Maximal RT value		Maximal p24 antigen value	
		Mean ± SD	Fold increase	Mean ± SD	Fold increase	Mean ± SD	Fold increase
207	Nef	14480 ± 707	7.8	19229 ± 3284	8.7	1408 ± 38	14667
	SEA	44040 ± 2424	19.4	26700 ± 3117	12.0	2541 ± 178	26469
	PHA	N.D.	-	N.D.	-	N.D.	-
	None	2389 ± 690	-	2118 ± 162	-	0.096 ± 0.01	-
208	Nef	19473 ± 3713	17.7	13133 ± 2083	19.7	434 ± 172	4667
	SEA	38033 ± 7971	33.6	14424 ± 318	21.7	520 ± 61	5591
	PHA	N.D.	-	N.D.	-	N.D.	-
	None	1167 ± 58	-	1009 ± 249	-	0.093 ± 0.01	-
209	Nef	3314 ± 830	4.3	1310 ± 291	1.3	51 ± 25	70
	SEA	20244 ± 1204	21.2	4558 ± 360	4.5	226 ± 81	310
	PHA	N.D.	-	N.D.	-	N.D.	-
	None	1004 ± 185	-	666 ± 130	-	0.73 ± 0.1	-

supernatants of cultures stimulated with Nef, SEA, and PHA. All supernatants were adjusted to achieve equivalent RT units per culture. The results of two representative experiments are shown in Figure 17. Supernatants from Nef-stimulated cultures had high titers of infectious virus similar to supernatants from cultures stimulated with SEA and PHA. These results indicate that virus replication in Nef-stimulated cultures, like the replication in SEA- and PHA-stimulated cultures, yielded infectious virus.

Nef-Stimulated PBMC Express T Cell Activation Markers

It has been shown that HIV requires activated T cells for virus replication. Cellular activation was investigated by double-staining FACS analysis of Nef-stimulated cells using antibodies to CD4 and HLA-DR (Table IX). Increased levels of dually-stained cells were observed in cultures of PBMC stimulated with Nef protein, as compared to unstimulated cultures. HLA-DR expression on $CD4^+$ human T cells has been shown to correlate with an activated T cell state. No increase was seen in cells that stained for CD8, either alone or in conjunction with HLA-DR. These results are consistent with our previous studies showing production of T helper cytokines upon stimulation with Nef. Thus, stimulation of PBMC with Nef protein induces activation of T helper cells.

Expression of Nef by HIV-Infected Cells

Culture supernatants of infected cells were tested for soluble Nef and none could be detected, suggesting that release of soluble Nef may not be occurring in these cultures. However, it has been shown that Nef is expressed on the surface of infected cells (Fujii et al., 1993). This leads to the question of whether Nef, in association with the extracellular membrane of infected cells, plays a role in the activation event(s) required for continuous HIV replication.

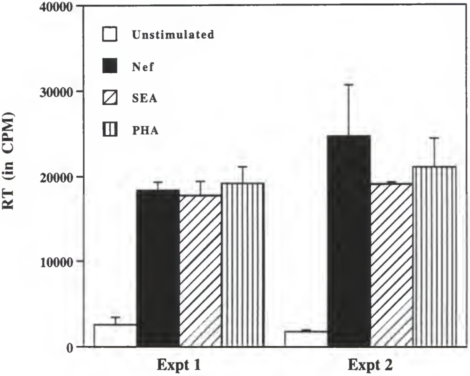

Figure 17. Infectivity assay on supernatants from Nef-stimulated HIV-infected cultures.

Table IX
Nef-induced activation of T cells as assessed by FACS

	% Total of dually-stained populations	
Cultures from:	CD4 + HLA-DR	CD4 + IL 2 R
Donor 206		
Unstimulated	3.17	N.D.[c]
Nef-stimulated	7.39	N.D.
SEA-stimulated	29.46	N.D.
Donor 213		
Unstimulated	6.65	N.D.
Nef-stimulated	16.74	N.D.
Donor 214		
Unstimulated	3.45	N.D.
Nef-stimulated	11.15	N.D.
Donor 215		
Unstimulated	N.D.	7.75
Nef-stimulated	N.D.	10.55
Donor 216		
Unstimulated	1.52	4.66
Nef-stimulated	9.02	7.68

Thus, we investigated the possibility that HIV-infected cells could activate autologous PBMC, similar to the activation seen with exogenously added Nef. To achieve maximal infection, PBMC were stimulated with mitogen prior to infection with HIV. After 5 days, HIV-infected cells were washed and inactivated with paraformaldehyde. Fresh autologous PBMC were cultured in the presence of infected cells and monitored for proliferation. Proliferation was observed in co-cultures of fresh and infected autologous cells (Table X). Uninfected stimulated cells served as controls, and no proliferation was observed in these co-cultures. Proliferative responses were significantly reduced by the addition of polyclonal anti-Nef antibodies, while control anti-SEA antibodies were without effect. Anti-Nef inhibition of proliferation was dose dependent, with lower concentrations of antibodies having less inhibitory activity. Similar to the results observed on HIV-infected cells, anti-Nef antibodies were capable of reducing proliferation in response to Nef, but not to SEA. Thus, a significant portion of the observed mitogenic activity of HIV-infected cells is due to Nef protein.

Discussion

It is interesting that HIV-infected cells were more potent at inducing T cell proliferation than was soluble Nef. Given that Nef interacts with MHC class II antigens, it is conceivable that optimal conditions for Nef association with MHC are achieved in HIV-infected cells, analogous to the expression of the mouse mammary tumor virus superantigen in infected B cells. These data suggest that proliferation occurred as a result of Nef expressed on the surface of HIV-infected cells, thereby indicating that Nef is a virally-encoded mitogen. Thus, the genome of HIV encodes for its own T cell mitogen, which would amplify the

Table X.
Ability of anti-Nef peptide antibodies to block proliferation induced by autologous HIV-infected cells

Fresh PBMC cultured in the presence of:	Anti-Nef antibodies	Proliferation		
		CPM (Mean ± SD)	Fold increase	p value
HIV-infected PBMC	-	137187 ± 1923	162.2	
HIV-infected PBMC	+	79740 ± 8764	94.3	<0.003
-	+	1973 ± 880	2.3	
-	-	846 ± 271	-	

replication of virus in the host. As such, Nef could be classified as a virulence factor for HIV.

It has been demonstrated that activated T cells are required for replication of HIV-1. On the other hand, the ability of HIV-1 to infect quiescent or resting cells is still questioned. In two studies using quiescent cells, HIV-1 replication *in vitro* was blocked at the level of virus entry (Gowda et al., 1989; Tang and Levy, 1990). Others have shown that blockage of replication occurred at an intracellular event, possibly at reverse transcription (Zack et al., 1990) or proviral integration (Stevenson et al., 1990; Bukrinsky et al., 1991). Upon entry into quiescent cells, replication could be induced by subsequent T cell activation. Consistent with data from *in vitro* studies are reports that high numbers of quiescent $CD4^+$ cells from asymptomatic HIV-infected patients harbor unintegrated viral DNA capable of integration subsequent to PHA stimulation. Further, AIDS patients have increased numbers of cells containing integrated virus, corresponding with greater production of virus at this stage of the disease. These findings indicate that HIV-1 may infect quiescent cells, but such infection is nonproductive until cell activation occurs.

Studies suggest that Nef plays an important role in HIV pathogenesis. Infection of adult rhesus monkey with a *nef*-deleted mutant of SIVmac239 resulted in low viral burden and did not cause disease, although infected animals were persistently seropositive (Daniel et al., 1992). Recently, genomic analysis of an HIV strain from a longterm survivor revealed a 118 bp deletion in the *nef* gene which resulted in an out-of-frame shift for downstream sequences (Kirchhoff et al., 1995). These studies hint at the importance of Nef in HIV pathogenesis in that repeated attempts to isolate virus from this patient with nonprogressive disease were unsuccessful. Mutations in *nef* may result in altered mitogenic activity of the protein, with concomitant reduction or loss of the ability to activate T cells. Possible consequences of nef mutations, then, would be a reduction both in the cellular reservoir for virus replication and in the viral load in the infected host. Other investigators have not found alterations in the *nef* gene in strains isolated from other longterm survivors (Cao et al., 1995). It would be interesting to determine the T cell reactivity of these individuals to Nef, as we have found that 10-15% of individuals that we tested did not respond to Nef. It is possible that nonresponsiveness to Nef may play an important role in longterm survival in some HIV-infected individuals. Thus, Nef may aid in the establishment and maintenance of infection, suggesting its role as a virulence factor for HIV.

CHAPTER VI
A MODEL FOR THE ROLE OF NEF IN THE PATHOGENESIS OF HIV

As shown in Chapters II-V, Nef binds to MHC class II antigens at a site(s) involved in bacterial superantigen binding. Like MMTV superantigen, Nef protein is encoded in the 3' long terminal repeat of the genome of lentiviruses such as HIV-1 and simian immunodeficiency virus (SIV). Nef induced significant levels of proliferation in PBMC from a wide sampling of donors (85-90%), although these responses are lower than those induced by SEA. Further, Nef stimulation resulted in the production of the T helper cell cytokines, IL 2 and interferon gamma (IFNγ). Proliferation in response to Nef was observed in reconstituted cultures consisting of T cells and inactivated APCs, which is compelling evidence for Nef superantigen activity. Further, Nef did not induce purified T cells to proliferate in the absence of APCs, which is consistent with Nef superantigen activity. However, no apparent Vβ expansions were found, although such data do not preclude the Vβ specificity of Nef activation. Thus, Nef has superantigen-like characteristics in that it binds to class II antigens, does not require processing by APC, and activates T cells to proliferate and release cytokines such as IL 2 and IFNγ.

The functional role that Nef plays in the lentiviral pathogenesis is uncertain, although its importance is reflected in vaccine studies in which a nef-deleted SIV mutant did not cause disease and protected animals upon challenge with the pathogenic wildtype strain (Daniel et al. 1992). The viral load in SIV mutant-infected animals was considerably lower than in animals challenged with wildtype SIV. Within this context, the ability of Nef to activate T cells is interesting in that active HIV replication requires T cell activation. Further, although resting or quiescent T cells can be infected, evidence suggests that HIV replication only occurs upon subsequent cellular activation.

Nef stimulation of PBMC was sufficient to induce HIV-1 replication. Virus replication in Nef-stimulated cultures yielded infectious virus, rather than defective particles. Flow cytometric analysis showed that increased percentages of cells that stained for both CD4 and HLA-DR were present in Nef-stimulated cultures. Further, increased percentages of cells that stained for both CD4 and IL 2R were observed in Nef-stimulated cultures. These results are consistent with the production of T helper cytokines upon stimulation with Nef. Thus, stimulation of PBMC with Nef protein induces T cell activation and such activation is sufficient for HIV replication.

Proliferation of fresh autologous PBMC cultured in the presence of infected cells was observed. Further, proliferative responses were significantly reduced by the addition of polyclonal anti-Nef antibodies. These data suggest that proliferation occurred as a result of Nef present on the surface of HIV-infected cells, thereby indicating that Nef may be a virally-encoded mitogen.

Nef has been shown to induce differentiation of human B cells to immunoglobulin secreting cells (Chirmule et al., 1994). Monoclonal antibodies to HLA-DR and adhesion molecules abrogated Nef-induced differentiation. B cell stimulation required T cells and monocytes, the latter producing IL 6 upon Nef stimulation. Interestingly, staphylococcal superantigens have been shown to induce B cell differentiation analogous to that described for Nef (Stohl et al., 1994). Thus, Nef can stimulate both T and B cells in a manner similar to the staphylococcal enterotoxin superantigens.

This leads to the question of the activation event(s) that occurs during HIV-1 exposure and that ultimately lead to the establishment of infection. Our results suggest a model in which Nef acts as a HIV-encoded T cell mitogen (Figure 18). Nef may interact with T cells either as an integral part of the membranes of infected T cells or in a soluble form released as the result of lysis of infected cells. Nef is present on the membranes of infected T cells (Fujii et al., 1993), which is consistent with this model. Nef expression may occur in the context of the T cell activation marker, HLA-DR, and the Nef/HLA-DR complex would stimulate uninfected T cells, as well as B cells. Such stimulation would allow for the expansion of a cellular reservoir for replication of the virus, eventually leading to T cell anergy and/or apoptosis. B cell activation and differentiation could ultimately lead to hypergammaglobulinemia. Thus, the HIV genome may encode for its own T cell mitogen, which would induce the amplification of virus replication in the host, with ultimate deleterious effects on the immune system. As such, Nef could be classified as a virulence factor for HIV.

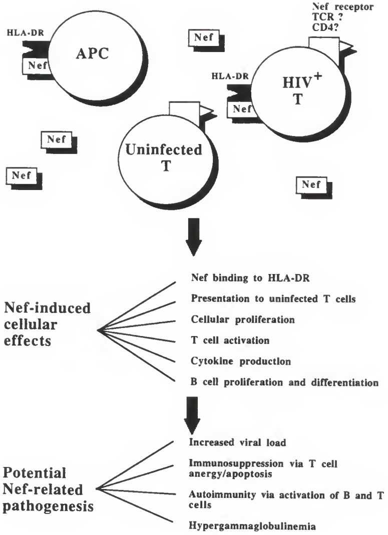
Nef receptor
TCR ?
CD4?
HLA-DR
APC
Nef
Nef
HLA-DR
HIV$^+$
T
Nef
Nef
Uninfected
T
Nef
Nef
Nef-induced cellular effects
Nef binding to HLA-DR
Presentation to uninfected T cells
Cellular proliferation
T cell activation
Cytokine production
B cell proliferation and differentiation
Potential Nef-related pathogenesis
Increased viral load
Immunosuppression via T cell anergy/apoptosis
Autoimmunity via activation of B and T cells
Hypergammaglobulinemia

Figure 18. Model for the role of Nef in the pathogenesis of HIV. Activation of uninfected T cells occurs by Nef interaction in either of two forms: as soluble Nef released from lysed cells, presented in the context of MHC class II antigens on APC, or as an integral part of the membranes of infected T cells, complexed to HLA-DR. Binding to uninfected T cells may occur via TCR or CD4. T cell activation results in proliferation and release of cytokines such as IFNγ and IL 2, thereby creating a cellular reservoir for virus and increasing viral load in the host. T cell activation results in depletion of T cells via virus production, anergy and/or apoptosis. B cell differentiation, possibly mediated by T cell cytokine release, results in hypergammaglobulinemia. Autoimmune-like sequelae may result from B cell differentiation into Ig-secreting cells, and activation of T cells.

LIST OF REFERENCES

Abe, J., B. L. Kotzin, K. Jujo, M. E. Melish, M. P. Glode, T. Kohsaka, and D. Y. M. Leung. 1992. Selective expansion of T cells expressing T-cell receptor variable region Vβ2 and Vβ8 in Kawasaki disease. Proc. Natl. Acad. Sci. USA 89:4066.

Acha--Orbea, H. 1992. Retroviral superantigens. In B. Fleischer, Ed. Ciological Significance of Superantigens. Basel: Karger, pp65-86.

Acha--Orbea, H. and E. Palmer. 1991. Mls - a retrovirus exploits the immune system. Immunol. Today 12:356.

Acha--Orbea, H., A. N. Shakhov, L. Scarpellino, E. Kolb, V. Muller, A. Vessaz-Shaw, R. Fuchs, K. Blochinger, P. Rollini, J. Billotte, M. Sarafidou, H. R. MacDonald, and H. Diggelmann. 1991. Clonal deletion of Vβ14-bearing T cells in mice transgenic for mammary tumor viurs. Nature 350:207.

Achong, B. G., P. W. A. Mansell, M. A. Epstein, and P. Clifford. 1971. An unusual virus in cultures from a human nasopharyngeal carcinoma. J. Nat. Cancer Inst. 46:299.

Allan, J. S., J. E. Coligan, T. --H. Lee, M. F. McLane, P. J. Kanki, J. E. Groopman, and M. Essex. 1985. A new HTLV-III/LAV encoded antigen detected by antibodies from AIDS patients. Science 230:810.

Arthur, L. O., J. W. Bess, R. C. Sowder, R. E. Benveniste, D. L. Mann, J. C. Chermann, and L. E. Henderson. 1992. Cellular proteins bound to immunodeficiency viruses: implications for pathogenesis and vaccines. Science 258:1935.

Baba, T. W., Y. S. Jeong, D. Penninck, R. Bronson, M. F. Greene, and R. M. Ruprecht. 1995. Pathogenicity of live, attenuated SIV after mucosal infection of neonatal macaques. Science 267:1820.

Bahraoui, E., M. Yagello, J. --N. Billaud, J. --M. Sabatier, B. Guy, E. Muchmore, M. Girard, and J. -C. Gluckman. 1990. Immunogenicity of the human immunodeficiency virus (HIV) recombinant nef gene product. Mapping of T-cell and B-cell epitopes in immunized chimpanzees. AIDS Res. Hum. Retroviruses 6:1087.

Barnstable, C. J., W. F. Bodmer, G. Brown, G. Galfre, C. Milstein, A. F. Williams, and A. Ziegler. 1978. Production of monoclonal antibodies to group A erythrocytes, HLA, and other human cell surface antigens - new tools for genetic analysis. Cell 14:9.

Bergdoll, M. S. 1985. The staphylococcal enterotoxins-an update. In J. Jeljaszewics, Ed. The Staphylococci. New York: Gustav Fisher Verlag, pp247-266.

Bisset, L. R., M. Opravil, E. Ludwig, and W. Fierz. 1993. T cell response to staphylococcal superantigens by asymptomatic HIV-infected individuals exhibits selective changes in T cell receptor V beta-chain usage. AIDS Res. Hum. Retroviruses 9:241.

Brodsky, F.M. 1984. A matrix approach to human class II histocompatibility antigens: reactions of four monoclonal antibodies with the products of nine haplotypes. Immunogenetics 19:179.

Buhrinsky, M. I., T. L. Stanwick, M. P. Dempsey, and M. Stevenson. 1991. Quiescent T lymphocytes as an inducible virus reservoir in HIV-1 infection. Science 254:423.

Cao, Y., L. Qin, L. Zhang, J. Safrit, and D. D. Ho. 1995. Virological and immunological characterization of long-term survivors of huma immunodeficiency virus type 1 infection. New Engl. J. Med. 332:233.

Carlsson, R., H. Fischer, and H. O. Sjogren. 1988. Binding of staphylococcal enterotoxin A to accessory cells is a requirement for its ability to activate human T cells. J. Immunol. 140:2484.

Chinatgumpala, M. M., J. A. Mollick, and R. R. Rich. 1991. Staphylococcal toxins bind to different sites on HLA-DR. J. Immunol. 147:3876.

Chirmule, N., N. Oyaizu, C. Saxinger, and S. Pahwa. 1994. Nef protein of HIV-1 has B-cell stimulatory activity. AIDS 8:733.

Choi, Y., J. Kappler, and P. Marrack. 1991. A superantigen encoded in the open reading frame of the 3' long terminal repeat of mouse mammary tumor virus. Nature 350:203.

Choi, Y., J. Kappler, and P. Marrack. 1992. Structural analysis of a mouse mammary tumor virus superantigen. J. Exp. Med. 175:847.

Cole, B. C. and M. M. Griffiths. 1993. Triggering and exacerbation of autoimmune arthritis by the *Mycoplasma arthriditis* superantigen MAM. Arth. Rheum. 36:994.

Dalgleish, A. G., S. Wilson, M. Gompels, C. Ludlam, B. Gazzard, A. M. Coates, and J. Habeshaw. 1992. T-cell receptor variable gene products and early HIV-1 infection. Lancet 339:824.

Daniel, M. D., F. Kirchhoff, S. C. Czajak, K. Sehgal, and R. C. Desrosiers. 1992. Protective effects of a live attenuated SIV vaccine with a deletion in the nef gene. Science 258:1938.

Dellabonna, P. J. Peccoud, J. Kappler, P. Marrack, C. Benoit, and D. Mathis. 1990. Superantigens interact with MHC class II molecules outside of the antigen binding groove. Cell 62:1115.

deRonde, A., B. Klaver, W. Keulen, L. Smit, and J. Goudsmit. 1992. Natural HIV-1 Nef accelerates virus replication in primary human lymphocytes. Virology 188:391.

Devereax, J., P. Haeberli, and O. Smithies. 1984. A comprehensive set of sequence analysis programs for the VAX. Nucl. Acid Res. 12:387.

Dobrescu, D., B. Ursea, M. Pope, A.S. Asch, and D.N. Posnett. 1995. Enhanced HIV-1 replication in Vβ12 due to human cytomegalovirus in monocytes: evidence for a putative herpesvirus superantigen. Cell 82:753.

Dyson, P. J., A. M. Knight, S. Fairchild, E. Simpson, and K. Tomonari. 1991. Genes encoding ligands for deletion of Vβ11 T cells cosegregate with mammary tumor virus genomes. Nature 349:531.

Edelman, A. S. and S. Zolla-Pazner. 1989. AIDS: a syndrome of immune dysregulation, dysfunction, and deficiency. FASEB J. 3:22.

Festenstein, H. 1973. Immunogenetic and biological aspects of *in vitro* lymphocyte allotransformation (MLR) in the mouse. Transplant. Rev. 15:62.

Fischer, H., M. Dohlsten, M. Lindvall, H. O. Sjogren, and R. Carlsson. 1989. Binding of staphylococcal enterotoxin A to HLA-DR on B cell lines. J. Immunol. 142:3151.

Fleischer, B. and H. Schrezenmeier. 1988. T cell stimulation by staphylococcal enterotoxins: Clonally variable response and requirement for major histocompatibility complex class II molecules on accessory or target cells. J. Exp. Med. 167:1697.

Fluegel, R. M. 1993. The molecular biology of the human spumavirus. In B. R. Cullen, Ed. Human Retroviruses. Oxford: IRL Press, pp193-214.

Franchini, G., M. Robert-Guroff, J. Ghrayeb, N. T. Chang, and F. Wong-Staal. 1986. Cytoplasmic localization of the HTLV-III 3' orf protein in cultured T cells. Virology 155:593.

Frankel, W. N., C. Rudy, J. M. Coffin, and B. T. Huber. 1991. Linkage of Mls genes to endogenous mammary tumor virus. Nature 349:525.

Fraser, J.D. 1989. High-affinity binding of staphylococcal enterotoxins A and B to HLA-DR. Nature 339:221.

Fujii, Y., Y. Nishino, T. Nakaya, K. Tokunaga, and K. Ikuta. 1993. Expression of human immunodeficiency virus type 1 Nef antigen on the surface of acutely and persistently infected human T cells. Vaccine 11:1240.

Gallo, R.C., Salahuddin, S.Z., M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, and B. Safai. 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science 224:500.

Garcia, J. V. and A. D. Miller. 1991. Serine-phosphorylation-independent downregulation of cell-surface CD4 by nef. Nature 350:508.

Golovkina, T. V., A. Chervonsky, J. P. Dudley, and S. R. Ross. 1992. Transgenic mouse mammary tumor virus superantigen prevents viral infection. Cell 69:637.

Gowda, S. D., B. S. Stein, N. Mohagheghpour, C. J. Benike, and E. G. Engleman. 1989. Evidence that T cell activation is required for HIV-1 entry in $CD4^+$ lymphocytes. J. Immunol. 142:773.

Griggs, N. D., C. H. Pontzer, M. A. Jarpe, and H. M. Johnson. 1992, Mapping of multiple binding domains of the superantigen staphylococcal enterotoxin A for HLA. J. Immunol. 148:2516.

Guy, B., M. P. Kierny, Y. Riviere, C. Le Peuch, K. Dott, M. Girard, L. Montagnier, and J. --P. Lecocq. 1987. HIV F/3' orf encodes a phosphorylated GTP-binding protein resembling an oncogene product. Nature 330:266.

Hammes, S. R., E. P. Dixon, M. H. Malim, B. R. Cullen, and W. C. Greene. 1989. *Nef* protein of human immunodeficiency virus type 1: evidence against its role as a transcriptional inhibitor. Proc. Natl. Acad. Sci. USA 86:9549.

Held, W., H. Acha--Orbea, H. R. MacDonald, and G. A. Waanders. 1994a. Superantigens and retroviral infections: insights from mouse mammary tumor virus. Immunol. Today 15:184.

Held, W., G. A. Waanders, H. R. MacDonald, and H. Acha--Orbea. 1994b. MHC class II hierarchy of superantigen presentation predicts efficiency of infection with mouse mammary tumor virus. Int. Immunol. 6:1403.

Held, W., G. A. Waanders, A. N. Shakhov, L. Scarpellino, H. Acha--Orbea, and H. R. MacDonald. 1993. Superantigen-induced immune stimulation amplifies mouse mammary tumor virus infection and allows transmission. Cell 74:529.

Herman, A., N. Labrecque, J. Thibodeau, P. Marrack, and R. R. Rich. 1991. Identification of the staphylococcal enterotoxin A superantigen binding site in the beta 1 domain of the human histocompatibility antigen HLA-DR. Proc. Natl. Acad. Sci. USA 88:9954.

Heston, W. E., M. K. Deringer, and H. B. Andervont. 1945. Gene-milk agent relationship in mammary tumor development. J. Natl. Cancer Inst. 5:289.

Ho, S. N., R. T. Abraham, S. Gillis, and D. J. Mckean. 1987. Differential bioassay of interleukin 2 and interleukin 4. J. Immunol. Methods. 98:99.

Hugin, A. W., M. S. Vacchio, and H. C. Morse III. 1991. A virus-encoded "superantigen" in a retrovirus-induced immunodefieciency syndrome of mice. Science 252:424.

Imberti, L., A. Sottini, A. Bettinardi, M. Puoti, and D. Primi. 1991. Selective depletion in HIV infection of T cells that bear specific T cell receptor Vβ sequences. Science 254:860.

Johnson, H. M. 1981. Cellular regulation of immune interferon production. Antiviral Res. 1:37.

Johnson, H. M. 1985 mechanism of Interferon-γ production and assessment of immunoregulatory properties. In E. Pick and M. Landy, Eds. Lymphokines Vol. 11. New York: Academic Press,pp 33-53.

Johnson, H. M. and J. A. Bukovic. 1975. Staphylococcal entertoxin A inhibition of the primary in vitro antibody response to thymus-dependent antigen. IRCS Med. Sci. 3:398.

Johnson, H. M., M. P. Langford, B. Lakhchaura, T. -S. Chan, and G. J. Stanton. 1982. Neutralization of native human gamma interferon (HuIFNγ) bt antibodies to a synthetic peptide encoded by the 5' end of HuIFNγ cDNA. J. Immunol. 129:2357.

Johnson, H. M. and H. I. Magazine. 1988. Potent mitogenic activity of staphylococcal enterotoxin A requires induction of interleukin 2. Int. Arch. Allergy Appl. Immunol. 87:87.

Johnson, H. M., J. K. Russell, and C. H. Pontzer. 1991. Staphylococcal enterotoxin superantigens. P.S.E.B.M. 198:765

Johnson, H. M., J. K. Russell, and C. H. Pontzer. 1992. Role of superantigens in human disease. Sci. Am. 266(4):92.

Johnson, H. M., B. A. Torres, and J. M. Soos. 1995. Superantigens : Structure and Relevance to Human Disease. Proc. Soc. Exp. Biol. Med. In Press.

Kappler, J., B. Kotzin, L. Herron, E. W. Gelfand, R. D. Bigler, A. Boylston, S. Carrel, D. N. Posnett, Y. Choi, and P. Marrack. 1989. Vβ-specific stimulation of human T cells by staphylococcal toxins. Science 244:811.

Kawabe,Y. and A. Ochi. 1990. Selective anergy of Vβ8$^+$ T cells in staphylococcus enterotoxin B-primed mice. J. Exp. Med. 172:1065.

Kawabe,Y. and A. Ochi. 1991. Programmed cell death and extrathymic reduction of Vβ8$^+$ CD4$^+$ T cells in mice tolerant to *Staphylococcus aureus* enterotoxin B. Nature 349:245.

Kirchhoff, F., T. C. Greenough, D. B. Brettler, J. L. Sullivan, and R. C. Desrosiers. 1995. Absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. New Engl. J. Med. 332:228.

Klatzmann, D., F. Barre-Sinoussi, M. T. Nugeyre, C. Danquet, E. Vilmer, C. Griscelli, F. Brun-Veziret, C. Rouzioux, J. C. Gluckman, J. C. Chermann, and L. Montagnier. 1984. Selective tropism of lymphadenopathy associated virus (LAV) for helper-inducer T lymphocytes. Science 225:59.

Kotzin, B. L/, D. Y. M. Leung, J. Kappler, and P. Marrack. 1993. Superantigens and their potential role in human disease. Adv. Immunol, 54:99.

Lafon, M., M. Lafage, A. Martinez-Arends, R. Ramirez, F. Vuiller, D. Charron, V. Lotteau, and D. Scott-Algara. 1992. Edidence for a viral superantigen in humans. Nature 358:507.

Lafon, M., D. Scott-Algara, P. N. Marche, P. A Cazenave. and E. Jouvin-Marche. 1994. Neonatal deletion and selctive expansion of mouse T cells by exposure to rabies virus nuceocapsid superantigen. J. Exp. Med. 180:1207.

Langford, M. P., G. J. Stanton, and H. M. Johnson. 1978a. Biological effects of staphylococcal enterotoxin A on human peripheral lymphocytes. Infect. Immun. 22:62.

Langford, M. P., D. A. Weigent, G. J. Stanton, and S. Baron. 1978b. Virus plaque-reduction assay for interferon. Microplaqye and regular macroplaque reduction assays. Methods Enzymol. 78:339.

Laurence, J., A. S. Hodstev, and D. N. Posnett. 1992. Superantigen implicated in dependence of HIV-1 replication in T cells on TCR Vβ expression. Nature 358:255.

Lechler, R. I., V. Bal, J. B. Rothbard, R. N. Germain, R. Sekaly, E. O. Long, and J. Lamb. 1988. Structural and functional studies of HLA-DR restricted antigen recognition by human helper T lymphocyte clones by using transfected murine cell lines. J. Immuno. 141:3003.

Lee, J. M. and T. H. Watts. 1990. Binding of staphylococcal enterotoxin A to purified murine MHC class II molecules in supported lipid bilayers. J. Immunol. 145:3360.

Lemaitre, M., D. Guetard, Y. henin, L. Montagnier, and A. Zerial. 1990. Protective activity of tetracycline analogs against the cytopathic effect of human immunodeficiency viruses in CEM cells. Res. Virol. 141:5.

Lewis, H. M., B. S. Baker, S. Bokth, A. V. Powles, J. J. Garioch, H. Valdimarsson, and L. Fry. 1993. Restricted T cell receptor Vβ gene usage in the skin of patients with guttate and chronic plaque psoriasis. Brit. J. Dermatol. 129:514.

Lifson, J. D., M. B. Feinberg, G. R. Reyes, L. Rabin, B. Banapour, S. Chakrabati, B. Moss, F. Wong-Staal, K. S. Steimer, and E. G. Engelman. 1986. Induction of CD4-dependent cell fusion by the HTLV-III/LAV envelope glycoprotein. Nature 323:725.

MacDonald, H. R., A. L. Glasebrook, R. Schneider, R. K. Lees, H. P. Pircher, T. Pedrazzini, O. Kanagawa, J.-F. Nicholas, R. C. Howe, R. M. Zinkernagel, and H. Hentgartner. 1989. T-cell reactivity and tolerance to Mls^{a}-encoded antigens. Immunol. Rev. 107:89.

Mariani, R. and J. Skowronski. 1993. CD4 down-regulation by nef alleles isolated from human immunodeficiency virus type 1-infected individuals. Proc. Natl. Acad. Sci. USA 90:5549.

Marrack, P. and J. Kappler. 1990. The staphylococcal enterotoxins and their relatives. Science 248:705.

Marrack, P., E. Kushnir, and J. Kappler. 1991. A maternally inherited superantigen encoded by a mouse mammary tumor virus. Nature 349:524.

Merryman, P., J. Silver, P. K. Gregeersen, G. Solomon, and R. Winchester. 1989. A novel association of DQ alpha and DQ beta genes in the DRw10 haplotype. J. Immunol. 143:2068.

Montagnier, L. 1995 Nef vaccination against HIV disease. Lancet 346:1170.

Mollick, J. A., R. G. Cook, and R. R. Rich. 1989. Class II molecules are specific receptors for staphylococcal enterotoxin A. Science 244:817.

Mottershead, D. G., P. -N. Hsu, R. G. Urban, J. L. Strominger, and B. T. Huber. 1995. Direct binding of Mtv7 superantigen (Mls-1) to soluble MHC class II molecules. Immunity 2:149.

Ohmen, J. D., P. F. Barnes, C. L. Grisso, B. R. Bloom, and R. L. Modlin. 1994. Evidence for a superantigen in human tuberculosis. Immunity 1:35.

Oroszlan, S. and R. B. Luftig. 1990. Retroviral proteinases. Curr. Top. Microbiol. Immunol. 157:153.

Ozato, K, N. M. Mayer, and D. H. Sachs. 1982. Monoclonal antibodies to mouse major histocompatibility complex antigens. Transplantation 34:113.

Parker, J. M. R., D. Guo, and R. S. Hodges. 1986. New hydrophilicity scale derived from high-performance liquid chromatography peptide retention data: correlation of predicted surface residues with antigenicity and x-ray-derived accessible sites. Biochem. 25:5425.

Peavy, D. L., W. H. Adler, and R. T. Smith. 1970. The mitogenic effects of endotoxin and staphylococcal enterotoxin B on mouse spleen cells and human peripheral lymphocytes. J. Immunol. 105:1453.

Poiesz, B. J., F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo. 1980. Detection and isolation of type C retrovirus particles from fresh cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc. natl. Acad. Sci. USA 77:7415.

Pontzer, C. H., N. D. Griggs, and H. M. Johnson. 1993. Agonist properties of a microbial superantigen peptide. J. Immunol. 193:191.

Pontzer, C. H., M. J. Irwin, N. R. J. Gascoigne, and H. M. Johnson. 1992. T cell antigen receptor binding sites for the microbial superantigen staphylococcal enterotoxin A. Proc Natl. Acad. Sci. USA 89:7727.

Pontzer C. H., J. K. Russell, and H. M. Johnson HM. 1991. Site of non-restrictive binding of SEA to class II MHC antigens. Int. Arch. Allergy Appl. Immunol. 93:107.

Pontzer, C. H., J. K. Russell, and H. M. Johnson. 1990. Site of nonrestrictive binding of SEA to class II MHC antigens. Int. Arch. Allergy Appl. Immunol. 93:107.

Pullen, A. M., Y. Choi, E. Kushnir, J. Kappler, and P. Marrack. 1992. The open reading frames inthe 3' long terminal repeats of several mouse mammary tumor virus integrants encode Vβ3-specific superantigens. J. Exp. Med. 175:41.

Rebai, N, G. Pantaleo, J. F. Demarest, C. Ciurli, H. Soudeyns, J. W. Adelsberger, M. Vaccarezza, R. E. Walker, R. P. Sekaly, and A. S. Fauci. 1994. Analysis of the T-cell receptor beta-chain variable-region (Vβ) repertoire in monozygotic twins discordant for human immunodeficiency virus: evidence for perturbations of specific V beta segments in CD4+ T cells of the virus-positive twins. Proc. Natl. Acad. Sci. USA 91:1529.

Rellahan, B. L., L. A. Jones, A. M. Kruisbeek, A. M. Fry, and L. A. Mathis. 1990. *In vivo* induction of anergy in peripheral Vβ8$^+$ T cells by staphylococcal enterotoxin B. J. Exp. Med. 172:1091.

Ringold, G.M. 1983. Regulation of mouse mammary tumor virus gene expression by glucocorticoid hormones. Curr. Top. Microbiol. Immunol. 106:79.

Robbins, P. A., E. L. Evans, A. H. Ding, N. L. Warner, and F. M. Brodsky. 1987. Monoclonal antibodies that distinguish between class II antigens (HLA-DP, DQ, and DR) in 14 haplotypes. Hum. Immunol. 18:301.

Rous, P. 1910. A transmissible avian neoplasm (sarcoma of common fowl). J. Exp. Med. 12:696.

Rous, P. 1911. Transmission of a malignant new growth by means of a cell-free filtrate. J. Amer. Med. Assoc. 56:198.

Russell, J. K., C. H. Pontzer, and H. M. Johnson. 1990. The I-Aβ^b region (65-85) is a binding site for the superantigen, staphylococcal entertoxin A. Biochem. Biophys. Res. Comm. 168:696.

Russell, J. K., C. H. Pontzer, and H. M. Johnson. 1991. Both α-helices along the major histocompatibility complex binding cleft are required for staphylococcal enterotoxin A function. Proc. Natl. Acad. Sci. USA 88:7228.

Salmons, B. and W. H. Gunzburg. 1987. Current perspectives in the biology of mouse mammary tumor virus. Virus Res. 8:81.

Schiffenbauer, J., H. M. Johnson, E. J. Butfilowski, L. Wegrzyn, and J. M. Soos. 1993. Staphylococcal enterotoxins can reactivate experimental allergic encephalomyelitis. Proc. Natl. Acad. Sci. USA 90:8543.

Sodrowski, J., W. C. Goh, C. Rosen, K. Campbell, and W. A. Haseltine. 1986. Role of the HTLV-III/LAV envelope in synctium formation and cytopathicity. Nature 322:470.

Soos, J. M. and H. M. Johnson. 1994. Type I interferon inhibition of superantigen stimulation: implications for treatment of superantigen-associated disease. J. Interferon and Cytokine Res. 15:39.

Spawski, J. B. and P. E. Lipsky. 1992. Isolation of B cell populations. In J. E. Coligan, A. M. Kruisbeek, D. H. Margulies, E. M. Shevach, and W. Strober, Eds. Protocols in Immunology. New York: John Wiley and Sons, p7.5.1.

Stevenson, M., S. Haggerty, C. Lamonica, A. M. Mann, C. Meier, and A. Wasiak. 1990. Cloning and characterization of human immunodeficiency virus type 1 variants diminished in the ability to induce syncytium-independent cytloysis. J. Virol. 64:3792.

Stohl, W., J. E. Elliott, and P. S. Linsley. 1994. Human T-cell dependent B cell differentiation induced by staphylococcal superantigens. J. Immunol. 153:117.

Tang, S. and J. A. Levy. 1990. Parameters involved in the cell fusion induced by HIV. AIDS 4:409.

Terwillegar, E. F., J. G. Sodroski, and W. A. Haseltine. 1990. Mechanisms of infectivity and replication of HIV-1 and implications for therapy. Ann. Emer. Med. 19:233.

Terwillegar, E. F., J. G. Sodroski, C. A. Rosen, and W. A. Haseltine. 1986. Effects of mutations within the 3' orf open reading frame region of human T-cell lymphotropic virus type III on replication and cytopathogenicity. J. Virol. 60:754.

Torres, B. A., J. K. Yamamoto, and H. M. Johnson. 1982. Cellular regulation of immune interferon (IFNγ) production: Lyt phenotype of the suppressor cell. Infect. Immun. 35:770.

Tsubura, A., M. Inabe, S. Imai, A. Murakami, N. Oyaizu, R. Yasumizu, Y. Ohnishi, H. Tanaka, S. Morii, and S. Ikehara. 1988. Intervention of T-cells in transportation of mouse mammary tumor virus (milk factor) to mammary gland cells *in vivo*. Cancer Res. 48:6555.

Uchiyama, T., T. Miyoshi-Akiyama, H. Kato, W. Fujimaki, K. Imanishi, and X. Yan. 1993. Superantigenic properties of a novel mitogenic substance produced by *Yersinia pseudotuberculosis* isolated from patients manifesting acute and systemic symptoms. J. Immunol. 151:4407.

Wain-Hobson, S., P. Sonigo, O. Danos, S. Cole, and M. Alizon. 1985. Nucleotide sequence of the AIDS virus LAV. Cell 40:9.

Winslow, G. M., M. T. Scherer, J. Kappler, and P. Marrack. 1992 Detection and biochemical characterization of the mouse mammary tumor virus-7 superantigen. Cell 71:719.

Woodland, D. L., M. P. Happ, K. J. Gollob, and E. Palmer. 1991. An endogenous retrovirus mediating deletion of αβ T cells? Nature 349:529.

Yoshida, M., I. Miyoshi, and Y. Hinuma. 1982. Isolation and characterization of retrovirus from cell lines of human T-cell leukemia and its implication in the disease. Proc. Natl. Acad. Sci. USA 79:2031.

Zack, J. A., S. J. Arrigo, S. R. Weitman, A. S.Go, A. Haislip, and I. S. Y. Chen. 1990. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. Cell 61:213.

Zinkernagel, R. and H. Hentgartner. 1994. T-cell mediated immunopathology versus direct cytolysis by virus: implications for HIV and AIDS. Immunol. Today 15:262.

BIOGRAPHICAL SKETCH

Barbara Aurea Torres, a first-generation American, was born on June 4, 1956 at Hialeah Hospital in South Florida. Her parents, Manuel and Antonia Torres, and sister, Marisol Torres Beaton, were born in Chile and emigrated to the United States in 1950. She was blessed with parents who intentionally gave her a first name that allowed her to "blend" into the American melting pot. Her father's family in Chile has had a long-time connection to medicine and the sciences. Barbara's grandfather was a physician in a rural community in Chile, and her uncle is still a general practitioner in Ecuador. In 1989, Barbara's father gave her a monograph on the atomic theory written by her great-grandfather, who was an instructor at the Sorbonne University in Paris, France. This monograph made a lasting impression on Barbara on the nature of scientific inquiry. Barbara graduated in 1978 with a Bachelor of Arts degree from Randolph-Macon Woman's College in Lynchburg, Virginia. In 1981, Barbara did graduate work in the laboratory of Dr. Howard M. Johnson and received a Master of Science degree from the Department of Microbiology at the University of Texas Medical Branch in Galveston, Texas. Barbara continued working with Dr. Johnson for several years, both in Galveston and at the University of Florida. After a mundane two-year stint at Bio-Rad Laboratories in Richmond, California, Barbara decided to return to Gainesville to work towards attaining her doctorate, once again in the laboratory of Dr. Johnson. After graduation, Barbara plans on continuing her research on HIV, as a postdoctoral fellow in the laboratory of Dr. Johnson at the University of Florida.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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